

1. Document ID: US 20010004757 A1

L1: Entry 1 of 24

File: PGPB

Jun 21, 2001

PGPUB-DOCUMENT-NUMBER: 20010004757
PGPUB-FILING-TYPE: new-utility
DOCUMENT-IDENTIFIER: US 20010004757 A1

TITLE: Processor and method of controlling the same

PUBLICATION-DATE: June 21, 2001

INVENTOR-INFORMATION:
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US-CL-CURRENT: 712/218; 712/226

AB: The present invention relates to a processor that performs a load operation prior to a store operation while avoiding ambiguous memory reference, and achieves high-speed operations. The present invention also relates to a method of controlling such a processor.

This processor includes a history control unit that stores a storage destination of a result obtained by executing a second instruction that is executed prior to a first instruction placed before the second instruction. When it is determined that the address of first data to be processed by the first instruction is included in the address region of second data to be processed by the second instruction, the history control unit overwrites the result obtained by the execution of the first instruction on the second data corresponding to the address.

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TITLE: Processor and method of controlling the same

BSTX:

[0009] Meanwhile, the register control unit 7 includes an EPCR register 31, an EPSR register 33, a PSR register 35, the general register 37, and the floating point register 39. The EPCR register 31, the EPSR register 33, and the PSR register 35 are connected to an interrupt control circuit 40. The general register 37 is connected to the load instruction execution unit 19, the store instruction execution unit 21, and the instruction execution circuit 23. The floating point register 39 is connected to the floating point load instruction execution unit 25, the floating point store instruction execution unit 27, and the floating point arithmetic operation instruction execution

unit 29.

BSTX:

[0018] When the instruction execution circuit 23 receives an arithmetic operation instruction from the instruction decoder unit 17, the instruction execution circuit 23 performs an operation based on a value read out from the general register 37, and writes the result in the general register 37. In a case where the instruction execution circuit 23 receives a branch instruction from the instruction decoder unit 17, the instruction execution circuit 23 supplies the branch destination address to the program counter 13 at the time of the occurrence of the branch. In a case where the instruction execution circuit 23 receives an interrupt return instruction, the instruction execution circuit 23 writes data that represents the pre-interrupt operation state in the register PSR 35. The instruction execution circuit 23 then reads the address of the instruction at the return destination from the register EPCR 31, and supplies the address as the branch destination address to the program counter 13. However, if the instruction execution circuit 23 detects an interrupt while executing the above instruction, the instruction execution circuit 23 supplies an interrupt signal to the interrupt control circuit 40.

BSTX:

[0019] The register EPCR 31 holds the address of an instruction corresponding to the return destination from the interrupt. The address is set at the occurrence of the interrupt. The register PSR 35 holds data that represents the operation state, and the register EPSR 33 holds data that represents the pre-interrupt operation state set prior to the occurrence of the interrupt.

BSTX:

[0020] Based on the interrupt signal supplied from the instruction read unit 3 or the instruction execution unit 5, the interrupt control circuit 40 writes the instruction address corresponding to the interrupt return destination in the register EPCR 31, the data that represents the pre-interrupt operation state in the register EPSR 33, and the operation state corresponding to the interrupt in the PSR 35. The interrupt control circuit 40 supplies the branch destination address corresponding to the interrupt to the instruction read unit 3.

BSTX:

[0022] When an interrupt occurs, the interrupt control circuit 40 writes the instruction address corresponding to the interrupt return destination in the register EPCR 31, the data that represents the pre-interrupt operation state in the EPSR 33, and the operation state of the interrupt in the PSR 35, based on the interrupt signal supplied from the instruction read unit 3 or the instruction execution unit 5. Also, the interrupt control circuit 40 supplies the branch destination address corresponding to the interrupt to the instruction read unit 3. The instruction read unit 3 then reads out an instruction word in accordance with the branch destination address supplied from the interrupt control unit 9, and supplies the instruction word to the instruction execution unit 5. After that, the operation is carried out in the same manner as in the normal state described above.

BSTX:

[0023] At the time of interrupt return, the instruction execution unit 5 executes an interrupt return instruction, thereby writing the value of the register EPSR 33 in the register PSR 35. The instruction execution unit 5 reads out the data from the register EPCR 31,

and supplies the result
as the branch destination address to the instruction read unit 3. The
instruction read unit 3 in
turn reads out an instruction word in accordance with the branch
destination address supplied from
the instruction execution unit 5, and supplies the instruction word to the
instruction execution
unit 5. After that, the operation is performed in the same manner as in the
above-described normal
state.

2. Document ID: US 6294368 B1

L1: Entry 2 of 24

File: USPT

Sep 25, 2001

US-PAT-NO: 6294368

DOCUMENT-IDENTIFIER: US 6294368 B1

TITLE: Isolated human metalloprotease proteins, nucleic acid molecules
encoding human protease
proteins, and uses thereof

DATE-ISSUED: September 25, 2001

INVENTOR-INFORMATION:

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US-CL-CURRENT: 435/219; 435/320.1, 435/69.1, 536/23.2, 536/23.5

AB: The present invention provides amino acid sequences of
peptides that are encoded by
genes within the human genome, the protease peptides of the present
invention. The present
invention specifically provides isolated peptide and nucleic acid
molecules, methods of
identifying orthologs and paralogs of the protease peptides, and methods
of identifying
modulators of the protease peptides.

L1: Entry 2 of 24

File: USPT

Sep 25, 2001

DOCUMENT-IDENTIFIER: US 6294368 B1

TITLE: Isolated human metalloprotease proteins, nucleic acid molecules
encoding human protease
proteins, and uses thereof

DEPR:

Full-length pre-processed forms, as well as mature processed forms, of
proteins that comprise one of
the peptides of the present invention can readily be identified as having
complete sequence identity
to one of the protease peptides of the present invention as well as being
encoded by the same
genetic locus as the protease peptide provided herein. As indicated by the
data presented in FIG. 3,
the map position was determined to be on chromosome 1 by ePCR.

DEPR:

Allelic variants of a protease peptide can readily be identified as being a
human protein having a
high degree (significant) of sequence homology/identity to at least a portion
of the protease
peptide as well as being encoded by the same genetic locus as the protease
peptide provided herein.
Genetic locus can readily be determined based on the genomic information
provided in FIG. 3, such as
the genomic sequence mapped to the reference human. As indicated by the
data presented in FIG. 3,
the map position was determined to be on chromosome 1 by ePCR. As
used herein, two proteins (or a
region of the proteins) have significant homology when the amino acid
sequences are typically at
least about 70-80%, 80-90%, and more typically at least about 90-95% or
more homologous. A
significantly homologous amino acid sequence, according to the present
invention, will be encoded by
a nucleic acid sequence that will hybridize to a protease peptide encoding
nucleic acid molecule
under stringent conditions as more fully described below.

DEPR:

Orthologs of a protease peptide can readily be identified as having some
degree of significant
sequence homology/identity to at least a portion of the protease peptide as
well as being encoded by
a gene from another organism. Preferred orthologs will be isolated from
mammals, preferably
primates, for the development of human therapeutic targets and agents.
Such orthologs will be
encoded by a nucleic acid sequence that will hybridize to a protease
peptide encoding nucleic acid
molecule under moderate to stringent conditions, as more fully described
below, depending on the
degree of relatedness of the two organisms yielding the proteins. As
indicated by the data presented
in FIG. 3, the map position was determined to be on chromosome 1 by
ePCR.

DEPR:

The nucleic acid molecules are also useful as probes for determining the
chromosomal positions of
the nucleic acid molecules by means of in situ hybridization methods. As
indicated by the data
presented in FIG. 3, the map position was determined to be on
chromosome 1 by ePCR.

DEPR:

Individuals carrying mutations in the protease gene can be detected at the
nucleic acid level by a
variety of techniques. FIG. 3 provides information on SNPs that have been
found in the gene encoding
the protease protein of the present invention. SNPs were identified at 7
different nucleotide
positions in introns and regions 5' and 3' of the ORF. Such SNPs in introns
and outside the ORF may
affect control/regulatory elements. As indicated by the data presented in
FIG. 3, the map position
was determined to be on chromosome 1 by ePCR. Genomic DNA can be

analyzed directly or can be amplified by using PCR prior to analysis. RNA or cDNA can be used in the same way. In some uses, detection of the mutation involves the use of a probe/primer in a polymerase chain reaction (PCR) (see, e.g. U.S. Pat. Nos. 4,683,195 and 4,683,202), such as anchor PCR or RACE PCR, or, alternatively, in a ligation chain reaction (LCR) (see, e.g., Landegran et al., Science 241:1077-1080 (1988); and Nakazawa et al., PNAS 91:360-364 (1994)), the latter of which can be particularly useful for detecting point mutations in the gene (see Abravaya et al., Nucleic Acids Res. 23:675-682 (1995)). This method can include the steps of collecting a sample of cells from a patient, isolating nucleic acid (e.g., genomic, mRNA or both) from the cells of the sample, contacting the nucleic acid sample with one or more primers which specifically hybridize to a gene under conditions such that hybridization and amplification of the gene (if present) occurs, and detecting the presence or absence of an amplification product, or detecting the size of the amplification product and comparing the length to a control sample. Deletions and insertions can be detected by a change in size of the amplified product compared to the normal genotype. Point mutations can be identified by hybridizing amplified DNA to normal RNA or antisense DNA sequences.

3. Document ID: US 6200751 B1

L1: Entry 3 of 24

File: USPT

Mar 13, 2001

US-PAT-NO: 6200751

DOCUMENT-IDENTIFIER: US 6200751 B1

TITLE: Endothelial specific expression regulated by EPCR control elements

DATE-ISSUED: March 13, 2001

INVENTOR-INFORMATION:

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US-CL-CURRENT: 435/6; 536/24.1

AB: The promoter of the EPCR gene has been isolated from both murine (SEQ. ID No. 1) and human (SEQ. ID No. 2) genomic libraries. The promoter includes a region (nucleotides 3130 to 3350 of SEQ. ID No. 1) which affects selective gene expression in endothelial cells), and a region (nucleotides 2270 to 2840 of SEQ. ID No. 1) which affects

selective gene expression in large vessel endothelial cells, as compared to expression in all endothelial cells. The EPCR promoter contains a thrombin responsive element, CCCACCCC (SEQ. ID No. 3), (murine, nucleotides 3007 to 3014 SEQ. ID No. 1 and human, nucleotides 2722 to 2729 SEQ. ID No. 2). The EPCR also contains a serum response element (nucleotides 2990 to 3061 of SEQ. ID No. 1). The regulatory sequences present in the EPCR promoter can be used for thrombin or serum controlled recombinant gene expression specific to either all endothelial cells or primarily endothelial cells of large vessels. Therapeutic strategies include the use of the minimal promoter for expression of therapeutic agents during times of increased thrombin/platelet activation or regional trauma in all endothelial cells or the use of the large vessel specific promoter for regional specific expression in the endothelial cells of large vessels for use in delivery.

L1: Entry 3 of 24

File: USPT

Mar 13, 2001

DOCUMENT-IDENTIFIER: US 6200751 B1
TITLE: Endothelial specific expression regulated by EPCR control elements

ABPL:

The promoter of the EPCR gene has been isolated from both murine (SEQ. ID No. 1) and human (SEQ. ID No. 2) genomic libraries. The promoter includes a region (nucleotides 3130 to 3350 of SEQ. ID No. 1) which affects selective gene expression in endothelial cells), and a region (nucleotides 2270 to 2840 of SEQ. ID No. 1) which affects selective gene expression in large vessel endothelial cells, as compared to expression in all endothelial cells. The EPCR promoter contains a thrombin responsive element, CCCACCCC (SEQ. ID No. 3), (murine, nucleotides 3007 to 3014 SEQ. ID No. 1 and human, nucleotides 2722 to 2729 SEQ. ID No. 2). The EPCR also contains a serum response element (nucleotides 2990 to 3061 of SEQ. ID No. 1). The regulatory sequences present in the EPCR promoter can be used for thrombin or serum controlled recombinant gene expression specific to either all endothelial cells or primarily endothelial cells of large vessels. Therapeutic strategies include the use of the minimal promoter for expression of therapeutic agents during times of increased thrombin/platelet activation or regional trauma in all endothelial cells or the use of the large vessel specific promoter for regional specific expression in the endothelial cells of large vessels for use in delivery.

PCPR:

This application claims the benefit of U.S. Provisional application Ser. No. 60/030,718 filed Nov. 8, 1996, by Charles T. Esmon, Wei Ding, Jian-Ming Gu and Kenji Fukudome, entitled "Thrombin Response Element", and U.S. Ser. No. 60/054,533 filed Aug. 4, 1997 by Charles T. Esmon and Jian-Ming Gu, entitled "Endothelial Specific Expression Regulated by EPCR Control Elements".

BSPR:

The promoter of the EPCR gene has been isolated from both murine (SEQ. ID No. 1) and human (SEQ. ID No. 2) genomic libraries. The promoter has been demonstrated to include a region which results in selective expression in endothelial cells, between -1 and -220 based on the positions relative to

the ATG encoding the first amino acid of the murine EPCR protein (nucleotides 3130 to 3350 of SEQ. ID No. 1), and a region which selectively results in expression in large vessel endothelial cells, as opposed to all endothelial cells, located between -700 and -1080 (nucleotides 2270 to 2840 of SEQ. ID No. 1). A thrombin responsive element has been identified in the EPCR promoter, from -337 to -345 in the murine promoter (nucleotides 3007 to 3014 SEQ. ID No. 1) and from -360 to -368 (nucleotides 2722 to 2729 SEQ. ID No. 2) in the human promoter. The sequence is CCCACCCC (SEQ. ID No. 3). A serum response element has also been identified between -280 and -350 (nucleotides 2990 to 3061 of SEQ. ID No. 1).

BSPR:
The regulatory sequences present in the EPCR promoter can be used in combination with genes encoding other proteins, as well as shorter oligonucleotides, to increase expression by exposure to thrombin or serum or to effect selective expression in endothelial cells generally or preferentially in endothelial cells of the large blood vessels. The gene control elements include elements responsive to environmental stimuli (either thrombin or serum); and information to determine distribution of the desired protein expression (large vessels). Therapeutic strategies include the use of the minimal promoter (-220 to -1) for expression in all endothelial cells, for example, for any kind of gene therapy where systemic distribution is desired; the use of a promoter including an environmental stimuli response element(s), for use in delivery of agents whose expression should be increased during times of increased thrombin/platelet activation or during regional trauma; the use of the minimal promoter including an environmental stimuli response element and the element directing expression to large vessel endothelium, where a response to regional trauma is desirable but only in large vessel endothelium, and the use of the minimal promoter and element directing expression to large vessel endothelium, where expression is specifically targeted to large vessel endothelium but is not increased in response to any particular stimuli.

DRPR:
FIGS. 1A-1B are a comparison of the nucleotide sequences of the murine EPCR (nucleotides 2993 to 3481 of SEQ ID No. 1) and human EPCR promoters (nucleotides 2704 to 3224 of SEQ. ID No. 2).

DRPR:
FIG. 4 is a schematic of the promoter. The top line indicates the structure of the promoter from -220 to -177, (showing nucleotides 3120 to 3157 of SEQ ID No. 1) which includes the transcription control elements required for constitutive expression in endothelial cells (nucleotides 3120 to 3156 of SEQ ID No. 1). AP4 and SP-1 are known promoter elements that bind proteins that control gene expression. The bottom line is a schematic representation of the EPCR promoter showing the locations of the large vessel specific element between -1080 and -700 ("C"), the element which includes the sequence responsible for thrombin induction ("B"), the endothelial specific region ("A"), and the EPCR encoding element. SP-1, AP-1 and AP-4 are known promoter elements which bind proteins involved in transcription control.

DEPR:
Specific targeting of expression of desired genes can be achieved through the selection and use of the regulatory sequences described herein in detail, isolated from the protein C receptor (EPCR). The protein C receptor is the first protein identified and reported with these

properties. It is expressed in high levels exclusively in large vessels, and the expression levels decrease with vessel size, until there is little-to-no expression detectable in capillaries.

DEPR:
The EPCR Regulator Sequences

DEPR:
The endothelial cell protein C binding protein (referred to herein as "EPCR") was cloned and characterized, as described in PCT/US95/09636 "Cloning and Regulation of an Endothelial Cell Protein C/Activated Protein C Receptor" Oklahoma Medical Research Foundation. The protein was predicted to consist of 238 amino acids, which includes a 15 amino acid signal sequence at the N-terminus, and a 23 amino acid transmembrane region which characterizes the receptor as a type 1 transmembrane protein. The protein binds with high affinity to both protein C and activated protein C (Kd=30 nM), which is a naturally occurring anticoagulant, and is calcium dependent.

DEPR:
Following identification and cloning of the endothelial cell protein C receptor (EPCR), it was determined that the EPCR was down regulated in cultured endothelial cells by TNF.alpha.. To determine the physiological relevance of this finding, EPCR mRNA levels in rats and mice challenged with LD.sub.95 levels of endotoxin were examined. Surprisingly, in response to endotoxin infusion, EPCR message rose within three hours to about four fold the basal level and remained elevated for twelve hours, then returning toward baseline at 24 hours. The rapid response suggested that a factor generated by endotoxin infusion could upregulate EPCR expression. Since thrombin is known to be one of these factors, rat microvascular cells in culture were treated with thrombin (0.1 units/ml). The cells exhibited a three to four fold increase in EPCR mRNA levels within three hours relative to control cells.

DEPR:
Physiologically, these results showing elevated mRNA levels three hours after exposure to thrombin, which begins to decline after twelve hours to baseline levels by 24 hours, are important since they suggest that thrombin plays a direct in vivo role in upregulation of EPCR expression. High level EPCR expression could contribute to the decrease observed in protein C levels during acute inflammatory response syndromes.

DEPR:
The gene encoding EPCR including the promoter region was then isolated from a murine genomic library, using the DNA encoding murine EPCR as a probe. A human genomic library was similarly screened with the DNA encoding human EPCR to isolate the promoter for the human EPCR. Analysis of the promoter revealed a thrombin response element. Gel shift assays revealed that thrombin treatment induced at least one factor that binds specifically to this promoter region. Further analysis yielded the sequence of the thrombin responsive element. This element can be used to increase selective expression in response to thrombin. The promoter is also selective in expression, with the EPCR being selectively expressed more in large vessel endothelial cells when most of the entire promoter is present, including the beginning region. When a shorter portion of the promoter is present, there is expression in all endothelial cells. These results are consistent with a repressor being present in the first part of the promoter which suppresses expression in capillary endothelial

cells.

DEPR:

Referring to FIGS. 1A-1B and SEQ. ID Nos 1 (the murine EPCR promoter) and 2 (the human EPCR promoter), the 5' regulatory sequences of the EPCR includes a transcription initiation promoter specific to endothelium contained in -1 to -220 (nucleotides 3130 to 3350 of SEQ. ID No. 1) (referred to for ease of reference as "A"), a control element responsive to thrombin (CCCACCCC) (SEQ. ID No. 3) located between -337 and -345 in the murine promoter (nucleotides 3007 to 3014 of SEQ. ID No. 1) and between -360 and -368 in the human promoter (nucleotides 2722 to 2729 of SEQ. ID No. 2) (referred to as "B"), a serum response element located between -280 and -350 (nucleotides 2990 to 3061 of SEQ. ID No. 1) (referred to as "D"), and a large vessel expression element located between -1080 and -700 (nucleotides 2270 to 2840 of SEQ. ID No. 1) (referred to as "C"). The latter directs expression primarily to large vessels such as aorta, coronary arteries, arteries and veins, rather than to capillaries.

DEPR:

FIGS. 1A-1B are a comparison of the sequences from the murine and human promoters, demonstrating that they are highly homologous. It is understood that the equivalent regions from the promoters of EPCR from other species could be used to achieve the same type of expression, and that sequences from different species could be used in combination, for example, A from the murine promoter and C from the human promoter.

DEPR:

The nucleotide sequences are important as hybridization probes, in selected expression of recombinant proteins other than EPCR, in increasing expression of recombinant proteins by exposure of the encoding construct to thrombin, and in design and screening of drugs and diagnostics for therapeutic and research purposes.

DEPR:

The constructs are particularly useful in gene therapy. The elements can be used to regulate expression of a gene encoding an important protein, or a biologically active nucleic acid molecule such as antisense, triplex forming molecules, ribozymes, and guide sequences for RNAase P which can be used to mutate or stop transcription of a particular gene. Examples of gene targeting include expression of thrombomodulin (TM), EPCR, TFPI, tPA, or heparin (heparan proteoglycans) in large vessel endothelium to decrease clot propensity at atheromas or in autoimmune diseases. If systemic elevations of tPA was desired, sequence A could be used on the gene. Endogenous gene expression could be suppressed by using sequence A, ABC or possibly AC, coupled to antisense to block expression of adhesion molecules to decrease leukocyte infiltration in atherosclerosis. The thrombin response element is significantly inducible in vivo, and should therefore be particularly useful in the treatment of patients with a history of constitutively elevated levels of thrombin, for example, particularly for expression of therapeutic genes in coronary arteries in patients with unstable angina.

DEPR:

Nucleotide sequences were determined for 8.8 kb of the genomic structure and 3.4 kb of the 5'-flanking region of the mouse EPCR (mEPCR) gene. RNase protection assay revealed six major transcription start sites clustered at -110 to -119 upstream of the translation

initiation site. A

series of 5'-promoter deletion fragments: mP3340, mP1120, mP700, mP350 and an SV40 control were fused to a luciferase reporter gene and transiently transfected into several cell types, bovine aorta endothelial cells (large vessel endothelial cells), rat heart endothelial cells which is mostly capillary endothelial cells (small vessel endothelial cells), and 293 kidney cells (non-endothelial cells).

DEPR:

Transgenic mice were developed using either the -350 to -1 or -1080 to -1 regions of the mouse EPCR promoter to drive the structural gene for green fluorescent protein (GFP) to determine the in vivo activity of the previously described promoter regions.

DEPR:

The promoter regions (-1080 and -350) of mouse EPCR gene were cloned into the pEGFP1 vector (Clontech), which already contains the structural gene for GFP. The fragments which contained the promoter region of mEPCR and GFP reporter gene were released by enzymes Eco47 III and Afl II from the constructs pEGFP350 and pEGFP1080. After purification, the DNA fragments were microinjected into the pronuclei of fertilized mouse eggs by standard methods. Mice were screened for the presence of the transgene by GFP specific PCR and Southern blotting by standard methods. Several transgenic lines were established from both promoter constructs.

DEPR:

GFP mRNA was constitutively expressed in these lines. The level of GFP mRNA expression was variable from significantly less than to higher than the endogenous EPCR expression. These data indicate that the ability to express a foreign structural gene under the control of these promoters will not be chromosome integration position dependent, although constitutive level of expression may be influenced by chromosomal positioning.

DEPC:

Example 3: In vivo Activity of the EPCR Promoter.

CLPR:

1. An isolated naturally occurring cell specific regulatory element present in the endothelial protein C receptor promoter which directs expression to human, bovine, rat or murine endothelial cells, wherein said regulatory element is contained in nucleotides 3130 to 3350 of SEQ ID NO:1 or is contained in nucleotides 2868 to 3087 of SEQ ID NO:2.

CLPR:

4. An isolated naturally occurring tissue specific regulatory element present in the endothelial protein C receptor promoter which preferentially directs expression to human, bovine, rat or murine large vessel endothelial cells, wherein said regulatory element is contained in nucleotides 2270 to 2840 of SEQ ID NO:1 or is contained in nucleotides 2008 to 2388 of SEQ ID NO:2.

CLPR:

7. An isolated inducible regulatory element present in the endothelial protein C receptor promoter which is inducible by exposure to serum wherein said regulatory element is contained in nucleotides 2990 to 3061 of SEQ ID NO:1 or is contained in nucleotides 2738 to 2808 of SEQ ID NO:2.

CLPR:

10. A construct for expression of a gene comprising a regulatory element selected from the group consisting of an isolated naturally occurring regulatory element present in

the endothelial protein

C receptor promoter which directs expression to human, bovine, rat or murine endothelial cells,
which is contained in nucleotides 3130 to 3350 of SEQ ID NO:1 an isolated naturally occurring regulatory element present in the endothelial protein C receptor promoter which preferentially directs expression to human, bovine, rat or murine large vessel endothelial cells, which is contained in nucleotides 2270 to 2840 of SEQ ID NO:1, or which is contained in nucleotides 2008 to 2388 of SEQ ID NO:2, and an isolated regulatory element present in the endothelial protein C receptor promoter which is inducible by exposure to serum, which is contained in nucleotides 2990 to 3061 of SEQ ID NO:1 or is contained in nucleotides 2738 to 2808 of SEQ ID NO:2.

CLPR:

13. The construct of claim 10 wherein the regulatory element comprises an isolated regulatory element present in the endothelial protein C receptor promoter which directs expression to human, bovine, rat or murine endothelial cells, which is contained in nucleotides 3130 to 3350 of SEQ ID NO:1, or which is contained in nucleotides 2868 to 3087 of SEQ ID NO:2, and an isolated regulatory element present in the endothelial protein C receptor promoter which preferentially directs expression to human, bovine, rat or murine large vessel endothelial cells, which is contained in nucleotides 2270 to 2840 of SEQ ID NO:1 or which is contained in nucleotides 2008 to 2388 of SEQ ID NO:2.

CLPR:

15. A method for controlling the expression of a gene comprising expressing the gene under the control of a regulatory element selected from the group consisting of an isolated naturally occurring regulatory element present in the endothelial protein C receptor promoter which directs expression to human, bovine, rat or murine endothelial cells, which is contained in nucleotides 3130 to 3350 of SEQ ID NO:1, or which is contained in nucleotides 2868 to 3087 of SEQ ID NO:2, an isolated naturally occurring regulatory element present in the endothelial protein C receptor promoter which preferentially directs expression to human, bovine, rat or murine large vessel endothelial cells, which is contained in nucleotides 2270 to 2840 of SEQ ID NO:1, or which is contained in nucleotides 2008 to 2388 of SEQ ID NO:2, and an isolated regulatory element present in the endothelial protein C receptor promoter which is inducible by exposure to serum, which is contained in nucleotides 2990 to 3061 of SEQ ID NO:1 or which is contained in nucleotides 2738 to 2808 of SEQ ID NO:2.

CLPR:

17. The method of claim 15 wherein the gene is expressed under the control of an isolated naturally occurring regulatory element present in the endothelial protein C receptor promoter which directs expression to human, bovine, rat or murine endothelial cells, which is contained in nucleotides 3130 to 3350 of SEQ ID NO:1, or which is contained in nucleotides 2868 to 3087 of SEQ ID NO:2, and an isolated regulatory element present in the endothelial protein C receptor promoter which preferentially directs expression to human, bovine, rat or murine large vessel endothelial cells, which is contained in nucleotides 2270 to 2840 of SEQ ID NO:1, or which is contained in nucleotides 2008 to 2388 of SEQ ID NO:2.

ORPL:

Fukudome, et al., "Molecular Cloning and Expression of Murine and Bovine Endothelial Cell Protein C/Activated Protein C Receptor (EPCR)," J. Biological Chemistry 270(10):5571-5577 (1995).

ORPL:

Gu, et al., "Functional Characterization of 5'-Flanking Region of the Mouse Endothelial Protein C Receptor (EPCR) Gene," Mol. Biol. Cell Supp. 8:228a, Abstract No. 1323 (1997).

4. Document ID: US 6180355 B1

L1: Entry 4 of 24

File: USPT

Jan 30, 2001

US-PAT-NO: 6180355

DOCUMENT-IDENTIFIER: US 6180355 B1

TITLE: Method for diagnosing and treating chronic pelvic pain syndrome

DATE-ISSUED: January 30, 2001

INVENTOR-INFORMATION:

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US-CL-CURRENT: 435/7.1; 435/7.8

AB: The present invention provides a superior method of diagnosing Chronic Pelvic Pain

Syndrome in men comprising measuring levels of cytokines in semen or components or fractions of

semen. The invention also provides a method of treating a condition associated with elevated

levels of a cytokine, such as TNF-.alpha., in semen or a component or fraction thereof,

comprising administering a therapeutically effective amount of an ant-cytokine compound or

composition, such as an anti-TNF-.alpha. compound or composition.

L1: Entry 4 of 24

File: USPT

Jan 30, 2001

DOCUMENT-IDENTIFIER: US 6180355 B1

TITLE: Method for diagnosing and treating chronic pelvic pain syndrome

DETL:

formation of prohormone fragment(s) by proteolysis of the mediated disease prohormone, and uses of the antibody including prophylactic and therapeutic methods to treat disease, and diagnostic assays

for determining the amount of the prohormone and prohormone fragments present in a patient's body.

5,700,909 Dec. 23, Prosaposin and cytokine- Prosaposin and peptide derivatives derived therefrom will promote neurite outgrowth in 1997 derived peptides *in vitro*. A peptide consensus sequence was determined by comparing the active neurite outgrowth-inducing saposin C peptide sequence with that of various hematopoietic and neurotrophic cytokines. These cytokine-derived peptides will promote the same processes as their corresponding cytokines. In addition, prosaposin and saposin C promote increased nerve cell myelination *ex vivo*. 5,700,788 Dec. 23, Ureido derivatives of Subject of the present invention are new ureido derivatives of naphthalenephosphonic 1997 naphthalenephosphonic acids having the following formula (I) [See Original Patent for Chemical Structure Diagram] acids (I) [See Original Patent for Chemical Structure Diagram] wherein each of *m* and *n*, which are the same, is an integer of 1 to 4; each of *p* and *q*, which are the same, is an integer of 1 to 3; and each of the R groups, which are the same, is a free or esterified phosphonic acid group; and the pharmaceutically acceptable salts thereof. 5,698,711 Dec. 16, Compounds containing This invention is directed to the pharmaceutical use of phenyl compounds, which are 1997 phenyl linked to aryl or linked to an aryl moiety by various linkages, for inhibiting tumor necrosis factor. The heteroaryl by an aliphatic- invention is also directed to the compounds, their preparation and pharmaceutical or heteroatom-containing compositions containing these compounds. Furthermore, this invention is directed to the linking group pharmaceutical use of the compounds for inhibiting cyclic AMP phosphodiesterase. 5,698,706 Dec. 16, Heterocyclic amides and Peptidyl derivatives having a SH or acyl S group and which are amides, primary amides or 1997 methods of use thioamides, have therapeutic utility via MMP or TNF inhibition. 5,698,579 Dec. 16, Cyclic amides Cyclic amides are inhibitors of tumor necrosis factor and can be used to combat cachexia, 1997 endotoxin shock, and retrovirus replication. A typical embodiment is 3-phenyl-3-(1-oxoisindolin-2-yl)propionamide. 5,698,564 Dec. 16, Diphenyl disulfide Diphenyl disulfide compounds having an inhibiting activity against the production of 1997 compounds Interleukin-1 beta (IL-1 beta) or the release of Tumor Necrosis Factor alpha (TNF alpha), which are useful in the treatment or prophylaxis of the diseases such as chronic rheumatism and sepsis are described. 5,698,518 Dec. 16, Method for regulating A method of treating patients to inhibit inflammation is disclosed. In the method, an 1997 inflammation and tumor effective amount of calmodulin, a calmodulin analogue or calmodulin receptor agonist is growth with calmodulin, administered to a patient to inhibit production of tumor necrosis factor and/or augment calmodulin analogues or elastase. In another method, an effective amount of calmodulin antagonist is administered calmodulin antagonists to a patient to stimulate immune response or inhibit elastase release. In another embodiment, a diagnostic test is disclosed to be used on patient blood samples to determine individual propensity to regulate tumor necrosis factor and/or elastase by calmodulin, its analogues or receptor agonists. 5,698,391 Dec. 16, Methods for synthetic Methods useful for the determination of oligomers which have specific activity for a target 1997 unrandomization of molecule from a pool of primarily randomly assembled oligomers are provided. The oligomer fragments disclosed methods involve repeated syntheses of increasingly simplified sets of oligomers coupled with selection procedures for determining oligomers having the

highest activity. Freedom from the use of enzymes allows the application of these methods to any molecules which can be oligomerized in a controlled fashion. 5,698,195 Dec. 16, Methods of treating Anti-TNF antibodies, fragments and regions thereof which are specific for human tumor 1997 rheumatoid arthritis using necrosis factor- alpha (TNF alpha) and are useful *in vivo* for diagnosis and therapy of a chimeric anti-TNF number of TNF alpha -mediated pathologies and conditions, including rheumatoid arthritis antibodies as well as polynucleotides coding for murine and chimeric antibodies, methods of producing the antibody, methods of use of the anti-TNF antibody, or fragment, region or derivative thereof, in immunoassays and immunotherapeutic approaches are provided. 5,695,993 Dec. 9, Cloning and regulation of Human protein C and activated protein C were shown to bind to endothelium specifically, 1997 an endothelial cell protein selectively and saturably ($K_d = 30 \text{ nM}$, 7000 sites per cell) in a Ca^{2+} dependent C/activated protein C fashion. Expression cloning revealed a 1.3 kb cDNA that coded for a novel type receptor transmembrane glycoprotein capable of binding protein C. This protein appears to be a member of the CD1/MHC superfamily. Like thrombomodulin, the receptor involved in protein C activation, the endothelial cell protein C receptor (EPCR) function and message are both down regulated by exposure of endothelium to TNF. Identification of EPCR as a member of the CD1/MHC superfamily provides insights into the role of protein C in regulating the inflammatory response, and determination of methods for pharmaceutical use in manipulating the inflammatory response. 5,695,953 Dec. 9, DNA that encodes a tumor Necrosis Factor (TNF) Inhibitory Protein is isolated and substantially purified and 1997 necrosis factor inhibitory the DNA that encodes the TNF inhibitory protein, vectors, host cells, and a recombinant protein and a recombinant method for producing the encoded protein are also set forth. It has the ability to inhibit: (a) method of production the binding of TNF to its receptors, and (b) the cytotoxic effect of TNF. TNF Inhibitory Protein and salts, functional derivatives and active fractions thereof can be used to antagonize the deleterious effects of TNF. 5,691,382 Nov. 25, Inhibition of TNF production The present invention is directed to the method of inhibiting the release of tumor necrosis 1997 with matrix factor (TNF) in a condition mediated by TNF by administration of certain hydroxamic add metalloproteinase inhibitors derivatives, also known as matrix metalloproteinase inhibitors, and thus the method of this invention is useful in the management of diseases or conditions mediated by TNF. 5,691,381 Nov. 25, Hydroxamic and The present invention provides novel hydroxamic acids and carbocyclic acids and 1997 carbocyclic acids as derivatives thereof and to pharmaceutical compositions and methods of use of these novel metalloprotease inhibitors compounds for the inhibition of matrix metalloproteinases, such as stromelysin, and inhibit the production of tumor necrosis factor alpha, and for the treatment of arthritis and other related inflammatory diseases. these novel compounds are represented by Formula I below [See Original Patent for Chemical Structure Diagram] Formula I 5,688,805 Nov. 18, Tricyclic derivatives, Disclosed are compounds of Formula [See Original Patent for Chemical Structure 1997 compositions and methods Diagram] (I) or a pharmaceutically acceptable salt or solvate thereof. Also disclosed are of use pharmaceutical compositions containing compounds of Formula I, methods for inhibiting tumor necrosis factor- alpha and methods for treating septic shock, inflammation, or

allergic disease. 5,686,455 Nov. 11, Imidazole derivatives and As cytokine inhibitors

2,4,5-triylimidazole compounds and compositions for use as 1997 their use as cytokine cytokine

inhibitors. inhibitors 5,686,431 Nov. 11, Methods of using low The present invention relates to

methods for the prevention and/or treatment of 1997 molecular weight heparins pathological processes involving the induction of TNF- alpha secretion comprising a for treatment of pathological

pharmaceutically acceptable carrier and a low molecular Weight heparin (LMWH). In the processes

pharmaceutical compositions of the present invention, the LMWH present in a low effective dose and

is administered at intervals of about 5-8 days. Furthermore, the LMWH is capable of inhibiting in

vitro TNF- alpha secretion by resting T cells and/or macrophages in response to T cell-specific

antigens, mitogens, macrophage activators, disrupted extracellular matrix (dECM), laminin,

fibronectin, and the like. 5,686,259 Nov. 11, Assay method for the Cleavage site blocking antibody

that binds to prohormones, preferable Tumor Necrosis 1997 detection of 26 kd TNF Factor, thereby

preventing the formation of prohormone fragment(s) by proteolysis of the prohormone prohormone, and

uses of the antibody including prophylactic and therapeutic methods to treat disease, and diagnostic

assays for determining the amount of the prohormone and prohormone fragments present in a patients

body. 5,684,222 Nov. 4, Mutant mouse having a The multiple biological activities of tumor necrosis

factor (TNF) are mediated by two 1997 disrupted TNFRp55 distinct cell surface receptors of 55 and 75

kDa. Mutant mice of the invention lacking tumor necrosis factor receptor (TNFR) p55 still express

functional TNFRp75 molecules at the cell surface. Normal weight and size of the mutant mice are not

altered. Thymocyte development and lymphocyte populations are normal, and clonal deletion of

potentially self-reactive T cells is not impaired. Activation of the nuclear transcription factor

kappa B (NF- kappa B), however, is completely abrogated after stimulation with TNF. Moreover,

TNFRp55 mutant mice are protected from septic shock induced by bacterial endotoxin or superantigen,

but Listeria clearance is severely impaired and mutant mice easily succumb to Listeria infection.

Thus, the two TNF receptors are not redundant, are independently controlled, and play different

roles in normal and pathological physiology. 5,679,696 Oct 21, Compounds containing This invention

is directed to the pharmaceutical use of phenyl compounds, which are 1997 phenyl linked to aryl or

linked to an aryl moiety by various linkages, for inhibiting tumor necrosis factor. The heteroaryl by

an aliphatic-or invention is also directed to the compounds, their preparation and pharmaceutical

heteroatom-containing compositions containing these compounds. Furthermore, this invention is

directed to the linking group pharmaceutical use of the compounds for inhibiting cyclic AMP

phosphodiesterase. 5,679,684 Oct. 21, Hydroxyalkylammonium- Novel hydroxyalkylammonium-pyrimidine of

the formula [See Original Patent for Chemical 1997 pyrimidines and nucleoside Structure Diagram] (I)

and nucleoside derivatives have been found to be useful as derivatives, useful as inhibitors of

inflammatory cytokines. They can be used, inter alia, in the therapy of septic inhibitors of

inflammatory shock, cachexia, rheumatoid arthritis, inflammatory bowel disease, multiple sclerosis

and cytokines AIDS. The compounds are typically prepared by reaction of an iodo substituted

nucleoside with the appropriately

5. Document ID: US 6091708 A

L1: Entry 5 of 24

File: USPT

Jul 18, 2000

US-PAT-NO: 6091708

DOCUMENT-IDENTIFIER: US 6091708 A

TITLE: Traffic shaper with multiply queued virtual paths

DATE-ISSUED: July 18, 2000

INVENTOR-INFORMATION:
NAME

CITY

STATE

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Matsunuma; Keiji

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N/A

N/A

JPX

US-CL-CURRENT: 370/233; 370/253, 370/397, 370/465

AB: A traffic shaper that feeds cells into an asynchronous-transfer-mode virtual-path-switching network has two queues per virtual path: a first queue providing a regular class of service, and a second queue providing a variable class of service. Cells are taken from the first queue in preference to the second queue, combined traffic from the two queues is kept within a peak cell rate, and traffic from the second queue is kept within an allowed cell rate. Forward resource-management cells are inserted at certain intervals in the cell flow taken from the second queue, and the allowed cell rate is adjusted according to backward resource-management cells received in reply. A third queue for priority cell traffic, limited to a priority cell rate lower than the peak cell rate, may also be provided.

L1: Entry 5 of 24

File: USPT

Jul 18, 2000

DOCUMENT-IDENTIFIER: US 6091708 A

TITLE: Traffic shaper with multiply queued virtual paths

DEPR:

Referring to FIG. 9, the priority queue 70 is controlled with reference to a priority cell rate parameter (EPCR) and a corresponding flag (FE) stored in the traffic control table 32. Each virtual path has a separate EPCR value and FE flag. The EPCR value is less than the peak cell rate PCR and, like the PCR value, can be set by software.

DEPR:

FIG. 10 shows the internal structure of the output scheduler 30 in the second embodiment, using the same reference numerals as in FIG. 3 for corresponding elements. The queue flag register 56 now stores three flag bits per virtual path, the QR and QV flags indicating the presence of cells in the

regular-class queue 22 and variable-class queue 24, and the QE flag indicating the presence of cells in the priority queue 70. The EPCR controller 72 in FIG. 10 manages the FE time-out flags according to the EPCR parameters, in the same way that the PCR controller 48 manages the FR flags according to the PCR parameters.

DEPR:

The operation of the second embodiment is illustrated in FIG. 11. A certain amount (EPCR) of the total bandwidth (PCR) of each virtual path is allocated to cells in the priority class (C=2). The rate at which priority cells are sent cannot exceed the limit set by the EPCR parameter. The part of the total bandwidth (PCR) not used by priority cell traffic is allocated to cells in the regular class (C=1). Any bandwidth remaining after that is allocated to cells in the variable class (C=0) and to FRM cells.

6. Document ID: US 6037450 A

L1: Entry 6 of 24

File: USPT

Mar 14, 2000

US-PAT-NO: 6037450

DOCUMENT-IDENTIFIER: US 6037450 A

TITLE: Diagnostic assays using soluble endothelial cell protein C/activated protein C receptor

DATE-ISSUED: March 14, 2000

INVENTOR-INFORMATION:

NAME

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ZIP CODE

COUNTRY

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Stearns-Kurosawa; Deborah J.

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N/A

Kurosawa; Shinichiro

Edmond
OK

N/A

N/A

US-CL-CURRENT: 530/350; 530/827, 530/830

AB: Plasma EPCR has been isolated, characterized and shown to block cellular protein C activation and APC anticoagulant activity. Plasma EPCR appears to be about 43,000 daltons and circulates at approximately 100 ng/ml (98.4 +/- 27.8 ng/ml, n=22). Plasma EPCR bound activated protein C with an affinity similar to that of recombinant soluble EPCR (Kd.sub.app approximately 30 nM), and inhibits both protein C activation on an endothelial cell line and APC anticoagulant activity in a one-stage factor Xa clotting assay.

Soluble plasma EPCR appears

to attenuate the membrane-bound EPCR augmentation of protein C activation and the anticoagulant function of activated protein C. Soluble EPCR has also been detected in urine. Levels of

soluble EPCR can rise in inflammatory disease associated with vascular injury and appear to be correlated with inflammation and disease states associated with abnormal coagulation. Since

EPCR expression is restricted to larger vessels and is usually negative in capillaries, these observations provide a mechanism for analyzing injury/stimulation of large vessel endothelial cells.

L1: Entry 6 of 24

File: USPT

Mar 14, 2000

DOCUMENT-IDENTIFIER: US 6037450 A

TITLE: Diagnostic assays using soluble endothelial cell protein C/activated protein C receptor

ABPL:

Plasma EPCR has been isolated, characterized and shown to block cellular protein C activation and

APC anticoagulant activity. Plasma EPCR appears to be about 43,000 daltons and circulates at approximately 100 ng/ml (98.4 +/- 27.8 ng/ml, n=22). Plasma EPCR bound activated protein C with an affinity similar to that of recombinant soluble EPCR (Kd.sub.app approximately 30 nM), and inhibits both protein C activation on an endothelial cell line and APC anticoagulant activity in a one-stage

factor Xa clotting assay. Soluble plasma EPCR appears to attenuate the membrane-bound EPCR augmentation of protein C activation and the anticoagulant function of activated protein C. Soluble

EPCR has also been detected in urine. Levels of soluble EPCR can rise in inflammatory disease

associated with vascular injury and appear to be correlated with inflammation and disease states associated with abnormal coagulation. Since EPCR expression is restricted to larger vessels and is usually negative in capillaries, these observations provide a mechanism for analyzing injury/stimulation of large vessel endothelial cells.

BSPR:

Endothelial cells play a critical role in the protein C pathway in that they express two of the

known receptors responsible for efficient protein C activation, thrombomodulin and the endothelial

protein C/APC receptor (EPCR) (Fukudome and Esmon. 1994. J. Biol. Chem. 269:26486-26491;

Stearns-Kurosawa, et al. 1996. Proc. Natl. Acad. Sci. (USA) 93:10212-10216). Thrombomodulin (CD141)

is a transmembrane cofactor that binds circulating thrombin with high affinity and the resultant

enzyme-cofactor complex is required for physiologically relevant protein C activation rates (Esmon

and Owen. 1981. Proc. Natl. Acad. Sci. (USA) 78:2249-2252; Dittman, W. A. 1991. Trends Cardiovasc.

Med. 1:331-336).

BSPR:

EPCR is a recently identified receptor with significant homology to the CD1/MHC class I family

(Fukudome and Esmon, 1994; Fukudome, et al. 1996. J. Biol. Chem. 271:17491-17498; Regan, et al.

1996. J. Biol. Chem. 271:17499-17503). The cloning and biological role of the endothelial cell

receptor for protein C was described in PCT/US95/09636 by Oklahoma Medical Research Foundation,

entitled "Cloning and Regulation of an Endothelial Cell Protein

C/Activated Protein C Receptor". The

protein was predicted to consist of 238 amino acids, which includes a 15 amino acid signal sequence at the N-terminus, and a 23 amino acid transmembrane region which characterizes the receptor as a type I transmembrane protein.

BSPR:

EPCR binds both protein C and APC with similar affinity (K_d sub. app. about 30 nM) (Fukudome, et al., 1996) in the presence of calcium and facilitates protein C activation by presenting the protein C substrate to the thrombin-thrombomodulin activation complex on cell surfaces (Stearns-Kurosawa, et al., 1996). Both endothelial cell receptors are type I transmembrane proteins in which the ligand binds to an extracellular domain and both have a short intracellular cytoplasmic tail (Fukudome, et al., 1996; Jackman, et al. 1987. *Proc. Natl. Acad. Sci. (USA)* 84:6425-6429; Wen, et al., 1987. *Biochemistry* 26:4350-4357; Suzuki, et al. 1987. *EMBO J.* 6:1891-1897). In addition, their *in vitro* cell surface expression is down-regulated similarly by tumor necrosis factor- α (Fukudome and Esmon 1994). However, the characteristics of soluble forms of thrombomodulin and EPCR differ in several respects. Recombinant soluble thrombomodulin has reduced cofactor activity relative to the membrane form (Galvin, et al. 1987. *J. Biol. Chem.* 262:2199-2205; Parkinson, et al. 1990. *J. Biol. Chem.* 265:12602-12610). With both purified components and with cells, the changes in thrombin's substrate specifically induced by thrombomodulin result from competition for a shared binding domain on thrombin as well as conformational alterations in the active site pocket (Ye, et al. 1991. *J. Biol. Chem.* 266:23016-23021; Lu, et al. 1989. *J. Biol. Chem.* 264:12956-12962; Ye, et al. 1992. *J. Biol. Chem.* 267:11023-11028; Hofsteenge, et al. 1986. *Biochem. J.* 237:243-251; Mathews, 1994. *Biochemistry* 33:13547-13552; Esmon, et al. 1982. *J. Biol. Chem.* 257:7944-7947; Sadler, et al. 1993. *Haemostasis* 23:183-193). Soluble thrombomodulin also accelerates inactivation of thrombin by a variety of inhibitors (Bourin and Lindahl. 1993. *Biochem. J.* 289:313-330; Rezaie, 1995. *J. Biol. Chem.* 270:25336-25339). Both plasma and urine contain detectable thrombomodulin (Takano, et al. 1990. *Blood*. 76:2024-2029; Ishii and Majerus. 1985. *J. Clin. Invest.* 76:2178-2181) and because the thrombomodulin gene does not contain introns (Jackman, et al., 1987), these soluble forms are due to proteolysis of the extracellular domain at the cell surface.

BSPR:

In contrast, recombinant soluble EPCR (rsEPCR), truncated just before the transmembrane domain, binds both protein C and APC with an affinity similar to that observed for intact cell-surface expressed EPCR (Fukudome, et al. 1996). APC anticoagulant activity is inhibited effectively when bound to rsEPCR (Regan, et al., 1996), presumably because both rsEPCR and factor Va share binding determinants in a groove reminiscent of the anion binding exosite I in thrombin occupied by thrombomodulin (Mather, et al. 1996. *EMBO J.* 15:6822-6831). However, rsEPCR does not appear to influence proteolysis of small synthetic substrates by APC, nor inactivation of APC by α -1-antitrypsin or protein C inhibitor (Regan, et al., 1996). Unlike membrane-bound EPCR which enhances protein C activation (Stearns-Kurosawa, et al., 1996), rsEPCR has little effect on protein C activation by the soluble thrombin-thrombomodulin complex (Regan, et al., 1996), suggesting that any soluble forms of EPCR might inhibit protein C activation by competing with membrane-associated EPCR for protein C.

BSPR:

Immunohistochemistry indicates that EPCR is present primarily on the surface of endothelial cells from large vessels and is absent or present at low levels on most capillary endothelial cells.

BSPR:

It is therefore an object of the present invention to identify therapeutic and diagnostic uses for naturally occurring soluble EPCR.

BSPR:

It is a further object of the present invention to characterize naturally occurring soluble EPCR.

BSPR:

Plasma EPCR (has been isolated, characterized and shown to block cellular protein C activation and APC anticoagulant activity. Plasma EPCR appears to be about 43,000 daltons and circulates at approximately 100 ng/ml (98.4 ± 27.8 ng/ml, $n=22$). Plasma EPCR was purified from human citrated plasma using ion-exchange, immunoaffinity, and protein C affinity chromatography. Flow cytometry experiments demonstrated that plasma EPCR bound activated protein C with an affinity similar to that previously determined from recombinant truncated EPCR (K_d sub. app. approximately 30 nM), defined as EPCR not including the transmembrane and cytoplasmic domains. Furthermore, plasma EPCR inhibited both protein C activation on an endothelial cell line and APC anticoagulant activity in a one-stage factor Xa clotting assay. Soluble EPCR has also been detected in human urine. Cloning of the gene encoding EPCR demonstrates that at least human EPCR can be alternatively spliced, yielding a truncated soluble EPCR including an insert unique to the alternatively spliced form (sEPCR). These results indicate that plasma EPCR can be derived either by proteolysis at the cell surface or by alternative splicing.

BSPR:

If the local concentrations of plasma EPCR are sufficiently high, particularly in disease states, the data indicates that the truncated soluble plasma EPCR could attenuate the membrane-bound EPCR augmentation of protein C activation and the anticoagulant function of activated protein C. As demonstrated by the examples comparing normal plasma EPCR with levels of EPCR from patients with an autoimmune disease (systemic lupus erythematosus, SLE) and sepsis (a disorder involving both inflammation and coagulation abnormalities), levels of soluble EPCR appear to be correlated with inflammation and disease states associated with abnormal coagulation. Assays are described based on measurement of soluble EPCR which are indicative of disease conditions involving coagulation, inflammation, and large vessel disease. Assay reagents are described, including isolated purified soluble EPCR, recombinant truncated soluble EPCR, and antibodies to the soluble EPCRs.

DRPR:

FIG. 1 is a schematic of the two known mechanisms for producing a soluble receptor as applied to EPCR, by proteolysis of the membrane-bound receptor to release an extracellular domain and leave the membrane anchor behind, and by alternative splicing of the mRNA, showing the sequences unique to membrane bound EPCR (mEPCR) and to proteolyzed plasma EPCR (pEPCR), and the sequence unique to soluble EPCR (sEPCR).

DRPR:

FIG. 3 shows the sequence inserted into human, bovine, murine, and baboon EPCR by alternative

splicing.

DRPR:

FIG. 4a is a graph showing that soluble plasma EPCR binds to human protein C and APC. EA.hy926 cells were incubated with 60 nM fl-APC in the presence of 0-500 nM rsEPCR (.circle-solid.) or plasma EPCR (.smallcircle.) for 30 minutes on ice. The cells were washed and cell-bound fluorescence was determined by flow cytometry as described. The intrinsic cell fluorescence in the absence of added fl-APC is indicated by the arrow. The mean cell fluorescence (MCF) plotted represents the average of duplicate MCF determinations.

DRPR:

FIGS. 4b and 4c are graphs showing soluble plasma EPCR and rsEPCR inhibit protein C activation on cell surfaces. In FIG. 4b, EA.hy926 cell monolayers were pre-incubated for 15 minutes at room temperature with 0.1 μ M protein C alone (.quadrature.) or with 1 μ M rsEPCR (.circle-solid.), or 2 μ g/ml 1494 mAb (.smallcircle.). Protein C activation was initiated by the addition of thrombin (2 nM final) and the reactions were stopped at the indicated times. Activated protein C was determined with an amidolytic assay and the activity rates in MOD/min are plotted for each time point. Control wells without added thrombin were included (.circle-solid.). Each data point represents the average of triplicate well determinations. In FIG. 4c, EA.hy926 cell monolayers were pre-incubated for 15 minutes at room temperature with 0.1 M protein C and the indicated concentrations of plasma EPCR (.smallcircle.) or rsEPCR (.circle-solid.). Thrombin (final 2 nM) was added and the activation proceeded for 60 minutes at room temperature. The supernatants were added to a mixture of antithrombin and heparin and activated protein C activities (MOD/min) were determined with an amidolytic assay. Each data point represents the average of triplicate well determinations.

DRPR:

FIG. 4d is a graph showing soluble plasma EPCR inhibits APC anticoagulant activity. The anticoagulant activity of APC (25 nM) was determined with a one-stage Xa clotting assay in the presence of 460 nM plasma EPCR or rsEPCR. The effect was reversed when either soluble EPCR was pre-incubated for 5 minutes with 42 μ g/ml of 1496 mAb which blocks binding of APC to EPCR. The data represent the average of 4-6 determinations \pm S.D.

DRPR:

FIG. 5 is a graph comparing levels of soluble plasma TM to soluble plasma PCR in lupus patients, demonstrating that there is no correlation between TM and EPCR values, but that the majority of lupus patients exhibit extremely elevated levels of soluble plasma EPCR.

DEPR:

Endothelial Protein C Receptor, EPCR.

DEPR:

Previous investigations into the function of EPCR found that protein C binding to the membrane form of EPCR resulted in facilitation of protein C activation by the thrombin-thrombomodulin complex on cell surfaces (Stearns-Kurosawa, et al., 1996), but that soluble recombinant EPCR inhibited APC anticoagulant activity (Regan, et al. 1996). These observations, along with the knowledge that soluble thrombomodulin degradation products in plasma are a marker of endothelial damage in various disease states, led to the question of whether a soluble circulating form(s) of EPCR existed and, if

so, what role it may have in the protein C pathway.

DEPR:

The examples demonstrate that a soluble form of EPCR circulates in plasma and is present in urine. In a healthy donor population, the plasma EPCR level was about 100 ng/ml and it appeared to be a single antigen species of approximately 43,000 daltons. Subsequent purification of the soluble EPCR from plasma and functional studies determined that it was capable of binding both protein C and APC with an affinity similar to intact membrane-bound EPCR. The in vitro studies using an endothelial cell line demonstrated that plasma EPCR inhibited protein C activation at near physiological concentrations of protein C and thrombin. In addition, direct addition of purified plasma EPCR to plasma resulted in inhibition of APC anticoagulant activity that was reversed with monoclonal antibodies to rsEPCR.

DEPR:

The identification of the purified plasma protein as being EPCR was based on comparison with the properties of rsEPCR. These proteins both reacted with the same battery of monoclonal and polyclonal antibodies, had the same amino-terminal sequence, bound to immobilized protein C in a Ca^{2+} -dependent fashion, and blocked protein C activation and APC anticoagulant activity with similar dose response curves. In addition, the affinities of both protein C and APC for rsEPCR and plasma EPCR are similar to the affinity of intact membrane-bound EPCR. These properties appear to be unique to EPCR.

DEPR:

Previous studies demonstrated that membrane-bound EPCR expressed on endothelial cells augments protein C activation by a factor of between three and five fold, whereas the examples demonstrate that the soluble form of EPCR purified from plasma inhibits protein C activation on endothelial cells and APC anticoagulant activity. This predicts that EPCR could modulate the protein C pathway in several ways. First, in the larger vessels where thrombomodulin concentration is low to the microcirculation, EPCR expression is correspondingly increased (Laszik, et al., Circulation 1997).

Immunohistochemistry shows that in most organs, EPCR expression is most intense on large vessels and decreases progressively with decreasing vessel size, with little or no expression in the most abundant endothelial cell type, the capillary endothelium. EPCR expression may play a critical role in capturing the protein C substrate from the circulation and presenting it to the thrombin-thrombomodulin complex for activation. This is supported by in vitro observations that both the EA.hy926 endothelial cell line and human umbilical vein endothelial cells have at least six times more surface-expressed EPCR antigen than thrombomodulin. In the microcirculation where thrombomodulin concentration is high and EPCR is low, one would predict little influence on protein C activation. Finally, circulating soluble EPCR may reduce the generation of APC and the ability of APC to inactivate factor Va.

DEPR:

In a healthy individual, the soluble EPCR levels are about 2.5 nM, a concentration well below both the K_d of APC (approximately 30 nM) and the 80 nM protein C concentration in the circulation. Both of the effects of soluble plasma EPCR (inhibition of APC anticoagulant activity and protein C activation) required considerably higher concentrations than that present in normal plasma, leaving

the question of the physiological role of the plasma EPCR uncertain.

Patients with soluble EPCR

levels that exceed 40 nM have been identified, as described in Example 3 (lupus). Thus, if the local concentration near the endothelial cell surface exceeds the systemic concentration, the soluble EPCR concentration would reach levels that would attenuate both APC generation and activity, contributing to thrombotic risk.

DEPR:

A soluble form of a receptor can be produced by proteolytic cleavage of the membrane-bound receptor or by alternative splicing mechanisms. Proteolysis at the membrane surface releases soluble thrombomodulin, and receptors for TNF, IL-1, IL-2, M-CSF, PDGF, and NGF (Heaney, et al. 1996. Blood 87:847-857). Soluble receptors have a multitude of potential functions including acting as antagonists of the membrane receptor, stabilizing the ligand, initiating ligand-mediated signaling, downmodulation of the membrane form, and binding to receptor inhibitors to indirectly facilitate receptor-ligand activity. The latter mechanism is used by the IL-1 receptor system in which the soluble isoforms of both IL-1 receptors are generated by proteolytic cleavage and tightly regulate the responsiveness to IL-1.alpha. and IL-1.beta. (Arend, et al. 1994. J. Immunol. 153:4766-4774).

The EPCR genomic structure contains an alternative splicing site which would code for a soluble protein truncated just before the transmembrane domain (Fukudome and Esmon. 1995. J. Biol. Chem. 270:5571-5577), as discussed below. Soluble IL-6 receptors appear to be generated by both

proteolytic and alternative splicing mechanisms (Mullberg, et al. 1994. J. Immunol. 152:4958-4968; Lust, et al. 1992. Cytokine 4:96-100; Horiuchi, et al. 1994. Eur. J. Immunol. 24:1945-1948). This

cleavage site can also be useful in recovering large quantities of soluble EPCR, by constructing an expression vector encoding the truncated EPCR immediately followed by a peptide sequence to which an antibody is specifically directed, as described in U.S. Pat. No. 5,298,599 to Morrissey and Esmon, the teachings of which are incorporated herein. The epitope will then be cleaved by proteolysis, before or after administration to a patient. See also U.S. Pat. No.4,782,137 to Hopp et al.

DEPR:

Immunohistochemical studies have indicated that EPCR is located primarily on endothelium of large vessels and is barely detectable in capillaries. Plasma EPCR derived from membrane-bound EPCR, can therefore serve as a marker of large vessel disease processes. Plasma EPCR may serve as a useful comparison with plasma thrombomodulin levels which have been shown to be modulated in a variety of disease states, but which would reflect both large and small vessel disease processes, but probably would be dominated by small vessel contributions since most endothelium is microvascular.

DEPR:

The cDNA for EPCR is predicted to code for a protein of 238 amino acids (Sequence ID No. 2), which includes a 15 amino acid signal sequence (von Heijne, (1986) Nucleic Acids Res. 14, 4683-4690) at the N-terminal. Therefore, the mature protein is predicted to contain 223 amino acids. Direct sequencing of the recombinant protein showed that the mature protein started at Ser18. Sequence ID

No. 2 is the predicted amino acid sequence of EPCR. Amino acids 1-15 of Sequence ID No. 2

(MLTTLPIILLSGWA) are the putative signal sequence determined by the method of von Heijne (von

Heijne, 1986). Amino acids 211-236 of Sequence ID No. 2

(LVLGVLVGGFIAGVAVGIFLCTGGR) are the

putative transmembrane domain. Potential N-glycosylation sites are present at amino acids 47-49, 64-66, 136-138, and 172-174 of Sequence ID No. 2. Extracellular cysteine residues are present at amino acids 17 (removed in plasma EPCR), 114, 118, and 186 of Sequence ID No. 2. A potential transmembrane region (Engelman et al., (1986) Annu. Rev. Biophys. Chem. 15, 321-53) consisting of 23 amino acids was identified at the C-terminal end (beginning at amino acid 211 of Sequence ID No. 2).

DEPR:

The protein is a type 1 transmembrane protein. The extracellular domain contains four potential N-glycosylation sites and three Cys residues. Glycosylation is not essential for activity, as shown by N-glycanase digestion. The cytoplasmic region contains only three amino acids and terminates with a Cys, which is palmitoylated. If the terminal cysteine is not properly palmitoylated, the protein may be secreted. Altering the sequence of the EPCR to replace this cysteine with another amino acid thereby provides a means for making an essentially full length EPCR which is secreted instead of being membrane bound.

DEPR:

Details of the following studies and results are described in the examples. Human plasma contains about 100 ng/ml of soluble EPCR (Table 1). This was measured by an enzyme linked immunoassay (ELISA) using two monoclonal antibodies (1494 mAb and 1495 mAb) and standard techniques. Significantly elevated soluble EPCR levels were found in patients with systemic lupus erythematosus and sepsis.

These levels seemed fairly high for a membrane-bound receptor that is present, with few exceptions, only on the surface of the large blood vessels. To put this in perspective, thrombomodulin (TM) is expressed on all endothelium, as well as some non-vascular cells, yet normal soluble TM levels are only about 10-40 ng/ml (Takano, et al., Blood 76:2024-2029, 1990). The soluble TM levels were elevated in the patients with lupus, but not sepsis. Importantly, there was no correlation between the plasma EPCR and TM levels in these patient groups ($r_{\text{sup.2}}=0.028$ and 0.034, respectively).

DEPR:

The lack of correlation between the plasma EPCR and TM levels and the high plasma EPCR concentration is consistent with the concept that plasma EPCR originates from both proteolytic and alternative splicing mechanisms. The genomic structure of human EPCR contains four exons, separated by introns.

Review of this sequence reveals an in-frame reading sequence after the exon III-intron III boundary (at the 5' GT) that includes a TAA stop codon at position 7527. Since this stop codon is upstream of exon IV that codes for the transmembrane domain, the predicted protein would contain a unique 48 residue carboxyl-terminal tail (coded for by the intron sequence) and would not contain a transmembrane anchor.

DEPR:

FIG. 1 is a diagram of two potential ways truncated EPCR can be derived: by proteolysis immediately before the transmembrane domain or by alternative splicing. As shown by FIG. 2, alternative splicing results in inclusion of a peptide sequence in the alternatively spliced truncated EPCR. As shown by

FIG. 3, this sequence is highly conserved between species, although slight differences exist, resulting in a new carboxyl-terminal tail of 48 residues for human and bovine EPCR, 51 residues for

murine EPCR, and 22 residues for baboon EPCR.

DEPR:

Patient samples can be screened for the presence of, and amount of, sEPCR or EPCR, using antibodies to either EPCR, the unique insert present in the alternatively spliced insert in EPCR, or antibodies which bind with greater affinity to either EPCR or sEPCR due to conformational differences. Samples can also be screened using other standard techniques to specifically quantitate proteins which are present.

DEPR:

Antibodies to EPCR, and in particular, soluble EPCR ("sEPCR"), and recombinant soluble EPCR ("rsEPCR") can be generated which are useful in detection, characterization or isolation of receptor proteins, as well as for modifying receptor protein activity, in most cases, through inhibition of ligand binding. Antibodies are generated by standard techniques, using human or animal purified or recombinant receptor proteins or fragments thereof as the immunogen.

DEPR:

Monoclonal antibodies to EPCR were obtained as described for other proteins by Esmon, et al., 1993. Methods Enzymol. 222:359-385. The antibodies referred to as 1494, 1495, and 1496 mAbs are IgG1.kappa. antibodies that bind to recombinant soluble EPCR and to cell surface-expressed EPCR. The 1494 and 1496 mAbs block the binding of protein C and APC to EPCR, and inhibit the ability of cellular EPCR to facilitate protein C activation by the thrombin-thrombomodulin complex. The 1495 mAb does not block ligand binding to EPCR, does not alter cell surface protein C activation, and has a binding epitope distinct from that for 1494 or 1486 mAb. The antibodies can be labelled using standard techniques, such as radiolabelling, enzyme labelling, fluorescent labels such as fluorescein, gold particles, dyes, and other means for detection of the antibodies. For example, antibody can be biotinylated with biotinamidocaproate N-hydroxysuccinimide ester using standard procedures. Antibody can be immobilized to a solid support for use in immunoassays, for example, AffiGel-10.TM., nitrocellulose, or microtiter wells, or use in solution phase immunoassays.

DEPR:

In a preferred embodiment, EPCR is measured using microtiter plates (Maxisorp.TM., NUNC NS, Roskilde, Denmark) coated with 50 microliters of 4 micrograms/ml 1495 mAb in 15 mM Na.sub.2 CO.sub.3, 35 mM NaHCO.sub.3, pH 9.6, at 4.degree. C. overnight. At room temperature, the plates are then washed three times with 20 mM Tris-HCl, 0.1 M NaCl, 0.05% Tween 20, pH 7.5 (assay buffer), and blocked with assay buffer containing 0.1% (wt/vol) gelatin for at least one hour. The wells are then washed, 50 microliter samples added in triplicate wells, and the plates incubated for one hour. The wells are aspirated, washed three times with assay buffer, and 50 microliters of 2 micrograms/ml biotin-1494 mAb added. The plates are incubated for 1 hour, washed three times, and 50 microliters of 0.25 micrograms/ml streptavidin-alkaline phosphatase conjugate (GIBCO BRL) added and incubated for an additional hour. The wells are washed five times, and the substrate and amplifier reagents from an ELISA amplification kit (GIBCO BRL) added sequentially at 15-min intervals according to the manufacturer's directions. The color development is stopped with 0.3 M H.sub.2 SO.sub.4, and the endpoint absorbance read at 490 nm on a V.sub.max microplate reader. Standards in triplicate wells are from 1.5 to 100 ng rsEPCR/ml in 20 mM Tris-HCl, 0.1 M NaCl, and 1

mM EDTA, 0.1% gelatin, pH 7.5.

The standard curve is linear from 1.5 to 12.5 ng/ml, and samples are diluted with the same buffer to fall within the linear range. Studies show that between one and two percent plasma does not affect the linearity of the assay or the sensitivity of the standard curve. Plasma samples from healthy volunteers were diluted with assay buffer containing 1 mM EDTA to a final 2% plasma, and EPCR antigen levels are calculated from the average of triplicate wells by reference to standard curve determined on the same plate.

DEPR:

The assay for soluble EPCR is useful in detection and analysis of coagulation and inflammatory states and disorders as discussed herein, such as autoimmune diseases like lupus, in transplant monitoring, sepsis, shock, pre-eclampsia, diabetes, cardiopulmonary bypass, unstable angina, restenosis, angioplasty (i.e., vascular disease), kidney or liver disease. For example, the EPCR is a marker for large blood vessels, and therefore for damage to large blood vessels. An increase in the amount of soluble EPCR is indicative of large vessel injury, resulting either in proteolysis of EPCR or stimulation of sEPCR synthesis. The ratio of EPCR to thrombomodulin can also be determined, based on either blood or urine samples, which is indicative of the relative extent of microvascular versus large vessel. The relative amounts of EPCR to cytokines, leukocyte activation markers and complement factors or activation markers can also be used to indicate disease state.

DEPR:

Since EPCR is present on endothelial cells, it is useful as a marker of endothelial cell damage. It can be used as an indicator of drug effect, both toxicity as well as efficacy. For example, in lupus patients, drugs effectively minimizing inflammatory/coagulation mediated, large vessel injury would result in decreasing EPCR levels.

DEPR:

The following abbreviations are used: rsEPCR, recombinant soluble EPCR with the HPC4 epitope inserted in place of the transmembrane domain and cytosolic tail; mAb, monoclonal antibody; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis.

DEPR:

An alternative assay was developed in which the coating and detecting antibodies were reversed (1494 mAb coating; biotin-1495 mAb detecting) and antibody binding was detected with the Blue Phos substrate (KPL Laboratories; Gaithersburg, Md.). This method was used to assay plasma EPCR in the sepsis patients. This assay was more sensitive, probably because of affinity differences, but both assays gave qualitatively similar results.

DEPR:

In the first preparation, plasma (1L) was diluted with an equal volume of 20 mM Tris-HCl, pH 7.5, 10 mM benzamidine, 400 units sodium heparin and batch-adsorbed for 1 hour with 1 g pre-swollen QAE resin. After settling, the resin was processed for purification of protein C (Esmon, et al. 1993). Solid ammonium sulfate was added to the supernatant at 4.degree. C. to 40% saturation, centrifuged, and additional ammonium sulfate was added to that supernatant to achieve 70% saturation. After centrifugation, the soft pellet was placed in dialysis bags and dialyzed overnight against 12 L of 20 mM Tris-HCl, 0.02% sodium azide, pH 7.4. The dialysate was applied to a 1496 mAb-AffiGel-10 immunoaffinity column (6 ml resin; 5 mg IgG/ml resin) equilibrated in 20

mM Tris-HCl, 0.1 M NaCl,

0.02% sodium azide, pH 7.4. The column was washed with more than 12 ml of the same buffer and eluted with 50% (v/v) ethylene glycol in 20 mM Tris-HCl, pH 7.4 (Jun Xu, unpublished observations). The

peak fractions from the elution were pooled (0.37 total OD.sub.280), concentrated (Centriprep 30,

Millipore), and the buffer exchanged to 20 mM Tris-HCl, 0.1 M NaCl, 3 mM CaCl.sub.2, 0.6 mM

MgCl.sub.2, 0.02% sodium azide, pH 7.4. This material was applied to a protein C affinity column

that had been previously prepared by applying the purified protein C (3 mg) to an HPC4-AffiGel-10

column (5 mg IgG/ml resin; 0.9.times.8 cm) in the same buffer. The HPC4 mAb binds the protein C

activation region in a calcium-dependent fashion (Esmon, et al. 1993; Stearns, et al. 1988) and does

not interfere with subsequent binding of EPCR to protein C. After applying the sample containing

plasma EPCR, the column was washed with approximately 12 ml of buffer and eluted with 20 mM

Tris-HCl, 0.1 M NaCl 5 mM EDTA, 10 mM MOPS, 0.02% sodium azide, pH 7.5. Fractions were monitored for

absorbance at 280 nm and for EPCR antigen using the ELISA described above. The eluate containing

both protein C and plasma EPCR was applied to an FPLC

(Pharmacia-LKB, Uppsala, Sweden) Mono Q column

and the column developed with a linear gradient of 0.1-1 M NaCl in 20 mM Tris-HCl, pH 7.5. About

half of the plasma EPCR did not bind to the Mono Q column, half eluted at about 0.2 M NaCl, and the

protein C eluted at approximately 0.5 M NaCl. Both ionic species of plasma EPCR appeared identical

on SDS-PAGE gels under reducing or non-reducing conditions with silver staining, with Coomassie BB

staining, or with gold staining (Pierce) after transfer to PVDF membranes, and on Western blots with

the biotin-polyclonal anti-rsEPCR antibody probe.

DEPR:

The second preparation of plasma EPCR was done starting with 4L of plasma to purify enough protein

for functional studies. In this case, the 1496-AffiGel-10 resin (20 ml of 5 mg IgG/ml resin) was

added directly to the citrated plasma, along with final concentrations of 10 mM benzamide, 1 mM

diisopropylfluorophosphate, and 0.5 units/ml sodium heparin. The plasma was batch-adsorbed overnight

at 4.degree. C. with gentle mixing. After the resin settled, the supernatant was processed for

protein C purification (Esmon, et al. 1993). The resin was packed into a 2.5.times.30 cm column,

washed extensively with 20 mM Tris-HCl, 0.1 M NaCl, 0.02% sodium azide, pH 7.4 and eluted with 50%

ethylene glycol in 20 mM Tris-HCl, pH 7.4. The eluate was pooled and concentrated (5.5 total

OD.sub.280), applied to a Mono Q column and the two ionic species (breakthrough and 0.2 M NaCl

eluate peak) were re-applied to the 1496-AffiGel-10 resin (1.5.times.11 cm). The column was eluted

with 50% ethylene glycol as before. The eluate (0.71 ODs) was concentrated and the buffer exchanged

to 20 mM Tris-HCl, 0.1 M NaCl, 3 mM CaCl.sub.2, 0.6 mM MgCl.sub.2, 0.02% sodium azide with a

Centriprep 30. This material was then applied to an affinity column in which protein C (2.9 mg) had

been initially applied in the same buffer to an HPC2-AffiGel-10 column (0.6.times.17 cm). The HPC2

mAb binds to the protein C serine protease domain and does not interfere with EPCR binding

(Fukudome, et al. 1996). The bound EPCR was eluted with buffer containing 5 mM EDTA. Contaminating

serum amyloid P (from the protein C sample) was removed by ion-exchange chromatography on the FPLC

Mono Q column. The sample was applied in 0.2 M NaCl, so that the plasma EPCR did not bind, and was

separated from the contaminants which eluted at 0.4-0.5 M NaCl. The resultant purified plasma EPCR

(0.193 OD.sub.280) appeared homogenous by SDS-PAGE with silver

staining and by Western blotting with

polyclonal anti-rsEPCR. This material was used for the functional studies and amino-terminal sequence analysis.

DEPR:

Plasma EPCR purity was determined from silver stained SDS-PAGE 10% gels and western blots of

membranes probed with biotin-goat anti-rsEPCR (reduced and non-reduced). A single band of

approximately 43,000 Da appears in both the serum and plasma samples after the membrane is probed

with the polyclonal antibody. The size of the protein detected appears slightly larger than the

rsEPCR. The other bands detected were background binding of IgG as judged by probing with preimmune

IgG and longer exposure times. overnight incubation of plasma samples with the anti-EPCR 1495 mAb

coupled to AffiGel-10 resin, followed by washing and elution of bound antigen under reducing

conditions, resulted in a single band detected by Western blotting with biotin-goat anti-rsEPCR

polyclonal antibody.

DEPR:

Determination of soluble EPCR antigen in plasma from healthy volunteers by ELISA using mAb 1495 as

the coating antibody found antigen levels of 91.1+/-24.5 ng/ml in females (n=12) and 107.2+/-30.2

ng/ml in males (n=10). When calculated together, the average plasma EPCR antigen level was

98.4+/-27.8 ng/ml. The value for males appeared to be slightly higher than for females, similar to

thrombomodulin (Quehenberger, et al. Thromb. Haemost. 76: 729-734), although the population studied

was too limited for statistical analysis and this study was not designed to assess differences due

to gender, age, diet or other variables.

DEPR:

Since the plasma EPCR appeared to be a single species at approximately 100 ng/ml, it became

important to determine whether the circulating EPCR could bind protein C and APC. Soluble EPCR was

purified from human plasma by a combination of ion-exchange chromatography, precipitation with

ammonium sulfate, and immunoadsorption by anti-EPCR 1496 mAb-AffiGel-10 column chromatography as

described in Experimental Procedures.

DEPR:

This plasma EPCR (approximately 110 .mu.g) was applied to a protein C affinity column prepared by

applying protein C (3 mg) to an anti-protein C HPC4 mAb-AffiGel-10 column in buffer containing 3 mM

CaCl.sub.2, 0.6 mM MgCl.sub.2. The column was washed and plasma EPCR was applied at fraction 19. The

column was washed and eluted with buffer containing 5 mM EDTA starting at fraction 35. Absorbance at

280 nm and EPCR antigen was determined for the fractions. EPCR antigen was determined by ELISA.

DEPR:

More than 98% of the applied plasma EPCR antigen bound to the protein C affinity column. The

absorbance profile indicates co-elution of EPCR and protein C from the antibody column, consistent

with the calcium-dependence of protein C binding to this antibody (Stearns, et al. 1988).

DEPR:

To purify sufficient protein for functional and structural studies, EPCR was purified from 4L of

plasma using a similar, but slightly modified procedure. After elution from a protein C-antibody

affinity column, residual contaminating proteins were removed by ion-exchange chromatography on an

FPLC Mono Q column. The resultant preparation of plasma EPCR appeared homogenous on SDS-PAGE 10% gels with silver staining and identical results were obtained with western blots probed with biotin-goat anti-rsEPCR polyclonal antibody under both reducing and non-reducing conditions.

Amino-terminal sequence analysis of the purified protein yielded only one sequence, S-Q-D-A-S-D, which is identical to the amino-terminal sequence of recombinant soluble EPCR (Sequence ID No. 2).

This is the first amino-terminal sequence determination of EPCR from a natural source.

DEPR:

The ability of plasma EPCR to bind to APC was assessed by competition studies in which plasma EPCR was allowed to compete with cellular EPCR for APC, and the resultant free APC that could bind to cellular EPCR was assessed by flow cytometry (FIG. 4a). APC labeled with fluorescein in the active site (β -APC) was incubated with EA.hy926 cells in the presence or absence of either plasma EPCR or rsEPCR. The EPCR concentration dependence for inhibition of APC binding to the cells was similar for both soluble forms of EPCR. This observation indicates that the affinity of plasma EPCR for binding APC is similar to that previously determined for the rsEPCR-APC binding interaction (K_d subapp approximately 30 nM).

DEPR:

While rsEPCR has little effect on protein C activation in a soluble system (Regan, et al. 1996), membrane-bound EPCR has a very potent ability to facilitate activation on cell surfaces (Stearns-Kurosawa, et al. 1996). The current data demonstrating the existence of a circulating form of EPCR capable of binding protein C and APC suggested that plasma EPCR has the potential to alter cell-surface activation of protein C. The thrombin-dependent activation of an approximately physiological level of protein C (0.1 μ M) on EA.hy926 cells was inhibited by excess rsEPCR almost to the level of that observed with the anti-rsEPCR 1494 mAb that blocks the EPCR-protein C binding interaction, as shown by FIG. 4b. Previous studies have demonstrated that rsEPCR has no effect on APC amidolytic activity using small synthetic substrates (Regan, et al. 1996). The plasma EPCR was slightly more effective in its ability to inhibit cell-surface protein C activation on the EA.hy926 cells relative to the rsEPCR, as shown by FIG. 4c.

DEPR:

In a one-stage factor Xa clotting assay, purified plasma and soluble recombinant EPCR inhibited the APC prolongation of clotting times similarly (FIG. 4d). Inhibition of APC anticoagulant activity by rsEPCR had been observed previously (Regan, et al. 1996). As expected, the 1496 mAb reversed this effect by blocking the APC-plasma EPCR binding interaction.

DEPR:

To address the question of whether soluble EPCR is present in urine, four urine samples were collected (first morning void) and analyzed for the presence of soluble EPCR by western blotting and ELISA.

DEPR:

Undiluted pediatric urine samples were compared to a 4% normal plasma and recombinant soluble EPCR (1 ng). The samples were incubated with biotin-goat-anti-rsEPCR and a streptavidin-alkaline phosphatase detection system.

DEPR:

The western blot indicates that a) soluble EPCR is present in urine, and b)

the soluble EPCR antigen

is present at a size similar to that observed in plasma. Obvious degradation is not observed. The amount of soluble EPCR in the four samples as quantified by ELISA was 40.3, 6.1, 35.6, and 90.1 ng/ml.

DEPR:

Normal human plasma EPCR concentration are about 100 ng/ml (98.4 \pm 27.8 ng/ml; 2.5 nM), as discussed above. A panel of samples from patients with lupus erythematosus (n=54) was assayed and soluble EPCR levels were found to range from non-detectable levels to greater than 1,700 ng/ml.

Fifteen patients had soluble EPCR levels greater than 200 ng/ml.

DEPR:

Previous studies have shown elevated soluble plasma TM levels in lupus patients due to endothelial damage and the current lupus patient samples were assayed for plasma TM as a reference. It was found that their soluble TM levels had absolutely no correlation with their soluble EPCR levels, as shown by FIG. 5. This is an important observation that suggests that the source of the soluble plasma EPCR is not simply from randomly damaged endothelium. In contrast to TM, membrane-bound EPCR expression in humans and primates is restricted primarily to the endothelium of large vessels, with capillaries expressing little EPCR. The distinctive localization of EPCR is expected to augment protein C activation locally to prevent large vessel thrombosis. The primary localization of membrane-bound EPCR to the large vessels points to a targeted thrombotic risk in the large vessels that may be predicted by soluble plasma EPCR concentrations.

DEPR:

Patient blood samples were taken at time 0 (entry into the Intensive Care Unit, ICU) and at two days and six days after treatment with anti-thrombin II (ATIII) or a placebo. Plasma soluble EPCR and soluble thrombomodulin (TM) were assayed only on time 0 samples.

DEPR:

These results are shown graphically in FIG. 6. As in the lupus patients, patients with sepsis show very significant elevations in plasma EPCR levels, not correlated with soluble TM levels.

DEPR:

The observation that soluble plasma EPCR inhibits both protein C activation and activated protein C anticoagulant activity indicates that the elevated plasma EPCR levels in these patients poses an additional thrombotic risk and marks evidence of vascular injury/responsiveness. Examples of conditions these are indicative of include disorders associated with endothelial cell stimulation, atherogenesis, leukocyte adhesion and plaque rupture.

DEPR:

As an initial approach to determine whether a soluble EPCR isoform could be generated by alternative splicing mechanism, RNA was isolated from human and baboon tissues and reverse transcriptase-PCR (RT-PCR) performed with gene-specific primers. Although the baboon EPCR genomic sequence is not known, primers based on the human sequence were used based on the reasoning that baboons and humans are closely related on the evolutionary scale.

DEPR:

In the RT-PCR procedure generally, total RNA is isolated from homogenized tissue. The RNA is mixed with a specific antisense primer, nucleotides and the reverse transcriptase enzyme. In the mix, the RNA serves as a template for the reverse transcriptase to create a first

strand cDNA. This new cDNA

template is then amplified by conventional PCR using specific primers and Taq polymerase. Primers that would amplify both the membrane form of EPCR (424 bp) and the predicted alternatively spliced

product (674 bp) were chosen. Products corresponding to both forms of EPCR were amplified from a variety of baboon tissues (FIG. 4) and human lung and placenta. Possible contamination with genomic

DNA was unlikely as judged by controls without reverse transcriptase and the lack of a 1,885 bp band in the reactions with the tissues.

DEPR:

The observation that the predicted soluble EPCR isoforms will have unique carboxyl terminal tails

provides a structural difference for distinguishing between the isoforms using isoform-specific

antibodies. The working model is that plasma levels of proteolyzed soluble EPCR will report

endothelial injury, whereas levels of alternatively spliced soluble EPCR will report an endothelial

response to stimuli. It is anticipated that the relative plasma levels of the soluble EPCR isoforms

will provide information on large vessel endothelial dysfunction and injury in specific pathologies.

DEPR:

RT-PCR products from human tissues: placenta, lung, and tongue, were electrophoresed using the CRES/CREA primers specific for EPCR. The procedures were the same as used for the baboon tissues.

Products corresponding to the membrane isoform of EPCR (mEPCR) and the alternatively-spliced soluble

EPCR isoform (sEPCR) were observed. The products look essentially the same as that seen using the

baboon tissues. The only difference is that the placental tissue appears to have additional products.

DEPL:

Detection and Characterization of Soluble EPCR; Physiological Role and Utility as a Marker

DEPL:

Nucleotide and Predicted Protein Structure Analysis of EPCR

DEPL:

Proteins. Human protein C (Esmon, et al. 1993. *Methods Enzymol.*

222:359-385), bovine thrombin (Owen,

et al. 1974. *J. Biol. Chem.* 249:594-605), and bovine antithrombin (Esmon

1977. "Factors regulating

the inhibition of thrombin by antithrombin III. In *Chemistry and Biology of Thrombin*". R. L.

Lundblad, J. W. Fenton, II, and K. G. Mann, editors. Ann Arbor Science, Ann Arbor. 403-411) were

purified as described. Recombinant soluble EPCR, rsEPCR, consists of the extracellular domain of

EPCR truncated at residue 210 just before the transmembrane domain, followed by a 12 residue

sequence that permits calcium-dependent immunoaffinity purification on the HPC4 monoclonal antibody

(Takahashi, et al. 1992; Stearns, et al. 1988. *J. Biol. Chem.* 263:826-832). The construction,

purification, and protein C/APC binding characteristics of rsEPCR

(Fukudome, et al. 1996). Goat preimmune serum and polyclonal antiserum to rsEPCR was prepared and the IgG purified (Fukudome, et

al. 1996). Goat anti-rsEPCR polyclonal antibody was biotinylated with biotinamidocaproate

N-hydroxysuccinimide ester using standard procedures.

DEPL:

Monoclonal antibodies. Monoclonal antibodies (mAb) against rsEPCR were obtained as described for

other proteins (Esmon, et al. 1993). The 1494, 1495, and 1496 mAb are IgG1k antibodies that bind to

rsEPCR and to cell surface-expressed EPCR. The 1494 and 1496 mAb block the binding of protein C and

APC to EPCR and inhibit the ability of cellular EPCR to facilitate protein C activation by the

thrombin-thrombomodulin complex (Stearns-Kurosawa, et al. 1996). The 1495 mAb does not block ligand

binding to EPCR, does not alter cell surface protein C activation and has a binding epitope distinct

from that for 1494 or 1496 mAb. The 1494 and 1495 mAbs were biotinylated with biotinamidocaproate

N-hydroxysuccinimide ester using standard procedures. The 1494 mAb was coupled to AffiGel-10,

according to the manufacturer's directions, for immunoaffinity purification of plasma EPCR. The

screening of anti-EPCR mAb was done using methods described by Stearns-Kurosawa, et al. (1996);

Fukudome, et al. (1996).

DEPL:

Clotting Assay. The effect of rsEPCR or purified plasma EPCR on APC (25 nM) anticoagulant activity

in a one-stage factor Xa clotting assay was performed (Regan, et al. 1996) in the presence or

absence of 83 .mu.g/ml 1496 mAb, an antibody that blocks APC-EPCR interaction (Stearns-Kurosawa, et

al. 1996). The soluble EPCRs and 1496 mAb were pre-incubated for 15 minutes before assay.

DEPL:

Flow Cytometric Analysis. To serve as a fluorescent probe, APC was labeled with fluorescein in the

active site (fl-APC) as described (Fukudome and Esmon, 1994; Bock, P. E. 1988. *Biochemistry*

27:6633-6639). The effect of rsEPCR or plasma EPCR on APC binding to EA.hy926 cells was studied by

flow cytometry (Fukudome, et al. 1996). Briefly, harvested cells were incubated for 30 min on ice

with 60 nM fl-APC in the absence or presence of increasing concentrations of either soluble EPCR

preparation, washed, and cell-bound fluorescence was determined by flow cytometry with 10,000 events

counted per sample. All assays were done in Hank's balanced salt solution supplemented with 1%

bovine serum albumin, 3 mM CaCl.sub.2, 0.6 mM MgCl.sub.2, and 0.02% sodium azide.

DEPL:

Cell surface protein C activation. EA.hy926 cells were cultured in 96-well tissue culture dishes

(Stearns-Kurosawa, et al. 1996). The confluent monolayers were washed three times with Hank's

balanced salt solution supplemented with 1% (w/v) bovine serum albumin, 3 mM CaCl.sub.2, 0.6 mM

MgCl.sub.2, and 0.02% sodium azide. All assays were done at room temperature in the same buffer in

60 .mu.l final volume, and all protein concentrations represent the final concentration in the

assay. Protein C was added (0.1 .mu.M) in the absence or presence of rsEPCR, plasma EPCR, or 1494

mAb at the indicated concentrations and pre-incubated with the cells for 15 minutes. Thrombin was

added to the mixtures (2 nM) to start the activation reactions. At the indicated time, 50 .mu.l

aliquots were removed and added to 10 .mu.l of antithrombin (0.7 .mu.M final) and heparin (5 U/ml

final) in a 96-well microtiter plate. APC amidolytic activity was determined by addition of

Spectrozyme PCA substrate (0.2 mM) and the rate of change in absorbance at 405 nm (mOD/min) was

measured on a Vmax kinetic microplate reader (Molecular Devices, Menlo Park, Calif.). All assay

points were done in triplicate wells and less than 10% of the protein C substrate was activated as

determined by reference to a standard curve of fully activated protein C versus mOD/min.

DEPL:

ELISA for quantitation of plasma EPCR. An enzyme-linked

immunosorbent assay for detection of EPCR

antigen in plasma was developed. Microtitre plates (Maxisorp; Nunc, Roskilde, Denmark) were coated with 50 .mu.l of 4 .mu.g/ml 1495 mAb in 15 mM Na.sub.2 CO.sub.3, 35 mM NaHCO.sub.3, pH 9.6 at 4.degree. C. overnight. The following steps were done at room temperature. The wells were washed three times with 20 mM Tris-HCl, 0.1 M NaCl, 0.05% Tween 20, pH 7.5 (assay buffer) and blocked with assay buffer containing 0.1% (w/v) gelatin for at least one hour. The wells were washed, 50 .mu.l samples were added in triplicate wells, and the plates were incubated for one hour. The wells were aspirated, washed three times with assay buffer and 50 .mu.l of 2 .mu.g/ml biotin-1494 mAb was added. The plates were incubated for one hour, washed three times and 50 .mu.l of 0.25 .mu.g/ml streptavidin-alkaline phosphatase conjugate (GibcoBRL) was added and incubated for an additional one hour. The wells were washed five times and the substrate and amplifier reagents from an ELISA amplification kit (GibcoBRL) were added sequentially at 15 minute intervals according to the manufacturer's directions. The color development was stopped with 0.3 M H.sub.2 SO.sub.4 and the endpoint absorbance at 490 nm was read on a Vmax microplate reader. Each plate contained standards in triplicate wells from 1.5-100 ng/ml rsEPCR in 20 mM Tris-HCl, 0.1 M NaCl, 1 mM EDTA, 0.1% gelatin, pH 7.5. The standard curve was linear (r=0.99) from 1.5-12.5 ng/ml and plasma samples were diluted with the same buffer to fall within the linear range. Preliminary experiments determined that a final concentration of 1-2% human plasma did not affect the linearity or sensitivity of the standard curve. Plasma samples from healthy volunteers were diluted with assay buffer containing 1 mM EDTA to a final 2% plasma and EPCR antigen levels were calculated from the average of triplicate wells by reference to a standard curve determined on the same plate.

DEPL:

Purification of plasma EPCR. Plasma EPCR as purified from human citrated plasma (Oklahoma Blood Institute) using a combination of ion-exchange chromatography, anti-rsEPCR mAb immunoaffinity chromatography, and chromatography on protein C affinity columns. Two preparations were done in slightly different ways.

DEPL:

Protein Sequencing. The amino-terminal sequence analysis of soluble plasma EPCR was performed in Dr. Kenneth Jackson's laboratory at the Molecular Biology Research Facility, W. K. Warren Medical Research Institute, Oklahoma City. Amino acids are designated by the standard one letter code.

DEPL:

A. EPCR ELISA: The coating antibody is 1494 mAb that binds to the ligand binding domain of EPCR. The detecting antibody is biotinylated 1495 mAb, which does not block protein C/APC binding, and does not cross-react with 1494 mAb. The detection system is streptavidin-alkaline phosphatase and BluePhos substrate (from KPL).

DEPC:

Identification of Functional Endothelial Protein C Receptor in Human Plasma

DEPC:

Detection of Soluble EPCR in Urine

DEPC:

Measurement of Plasma EPCR from Lupus Patients

DEPC:

Plasma Soluble EPCR in Septic Shock Patients

DEPC:

Identification of Alternatively Spliced Forms of EPCR in Baboon and Human Tissues

DETL:

TABLE 1 _____ Plasma soluble receptor levels Plasma EPCR Plasma TM

ng/ml	ng/ml	Normal
volunteers, 133.4 .+-. 53.4	*35.5 .+-. 20.4	n = 20
Systemic lupus erythematosus, (P = 0.0004)	262.1 .+-. 154.5*	104.7 .+-. 77.5*
0.0008) n = 40	Sepsis, n = 24	224.9 .+-. 74.5*
		39.9 .+-. 73.1 (P = 0.00009)

*Significant difference between the means relative to normal; unpaired Student's t test.

CLPR:

1. A modified endothelial protein C receptor having the structure of the protein defined by Seq. ID No. 2, residues 16-238 wherein the carboxyl terminal cysteine residue is replaced with another amino acid or is not palmitoylated.

CLPR:

2. The modified endothelial protein C receptor of claim 1 which is not glycosylated.

CLPR:

3. The modified endothelial protein C receptor of claim 1 further comprising a signal sequence having the structure of Seq. ID No. 2, residues 1-15.

CLPR:

4. The modified endothelial protein C receptor of claim 1 encoded by Seq. ID No. 1, or a nucleotide sequence hybridizing thereto under stringent conditions.

CLPR:

5. The modified endothelial protein C receptor of claim 4 encoded by Seq. ID No. 1.

CLPR:

6. The modified endothelial protein C receptor of claim 1 wherein the carboxyl terminal cysteine residue is replaced with another amino acid.

CLPR:

7. The modified endothelial protein C receptor of claim 1 wherein the carboxyl terminal cysteine residue is not palmitoylated.

CLPR:

8. The modified endothelial protein C receptor of claim 1 which is human endothelial protein C receptor except for the modification.

CLPR:

9. An isolated naturally occurring alternatively spliced endothelial protein C receptor present in plasma having the structure defined by Seq. ID No. 2, further comprising a protein insert after Gly 201.

CLPR:

10. The isolated naturally occurring alternatively spliced endothelial protein C receptor of claim 9 comprising protein inserts as shown in FIG. 3.

CLPR:

11. An isolated naturally occurring soluble endothelial protein C receptor having the structure of Seq. ID No. 2, residues 16-201, wherein the receptor has been cleaved at a naturally occurring proteolytic cleavage site before the transmembrane domain present in plasma.

CLPR:
12. The isolated naturally occurring soluble endothelial protein C receptor of claim 11 encoded by a DNA sequence hybridizing to Seq. ID No. 1 under stringent conditions.

ORPL:
Kurosawa, et al., "Identification of functional Endothelial Protein C Receptor in Human Plasma," J. Clin. Invest. 100(2): 411-418 (1997).

ORPL:
Fukudome and Esmon, "Molecular Cloning and Expression of Murine and Bovine Endothelial Cell Protein C/Activated Protein C Receptor (EPCR)--The Structural and Functional Conservation in Human, Bovine and Murine EPCR*," J. Biol. Chem. 270(10):5571-5577 (1995).

7. Document ID: US 5852171 A

L1: Entry 7 of 24

File: USPT

Dec 22, 1998

US-PAT-NO: 5852171

DOCUMENT-IDENTIFIER: US 5852171 A

TITLE: Cloning and regulation of an endothelial cell protein C/activated protein C receptor

DATE-ISSUED: December 22, 1998

INVENTOR-INFORMATION:

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N/A

US-CL-CURRENT: 530/350; 530/380

AB: Human protein C and activated protein C were shown to bind to endothelium specifically, selectively and saturably ($K_d=30$ nM, 7000 sites per cell) in a $Ca_{sup.2+}$ dependent fashion. Expression cloning revealed a 1.3 kb CDNA that coded for a novel type I transmembrane glycoprotein capable of binding protein C. This protein appears to be a member of the CD1/MHC superfamily. Like thrombomodulin, the receptor involved in protein C activation, the endothelial cell protein C receptor (EPCR) function and message are both down regulated by exposure of endothelium to TNF. Identification of EPCR as a member of the CD1/MHC superfamily provides insights into the role of protein C in regulating the inflammatory response, and determination of methods for pharmaceutical use in manipulating the inflammatory response.

L1: Entry 7 of 24

File: USPT

Dec 22, 1998

DOCUMENT-IDENTIFIER: US 5852171 A

TITLE: Cloning and regulation of an endothelial cell protein C/activated protein C receptor

ABPL:

Human protein C and activated protein C were shown to bind to endothelium specifically, selectively and saturably ($K_d=30$ nM, 7000 sites per cell) in a $Ca_{sup.2+}$ dependent fashion. Expression cloning revealed a 1.3 kb CDNA that coded for a novel type I transmembrane glycoprotein capable of binding protein C. This protein appears to be a member of the CD1/MHC superfamily. Like thrombomodulin, the receptor involved in protein C activation, the endothelial cell protein C receptor (EPCR) function and message are both down regulated by exposure of endothelium to TNF. Identification of EPCR as a member of the CD1/MHC superfamily provides insights into the role of protein C in regulating the inflammatory response, and determination of methods for pharmaceutical use in manipulating the inflammatory response.

BSPR:

An endothelial cell protein C binding protein (referred to herein as "EPCR") has been cloned and characterized. The protein is predicted to consist of 238 amino acids, which includes a 15 amino acid signal sequence at the N-terminus, and a 23 amino acid transmembrane region which characterizes the receptor as a type 1 transmembrane protein. The protein binds with high affinity to both protein C and activated protein C ($K_d=30$ nM) and is calcium dependent. The message and binding function of the receptor are both down regulated by cytokines such as TNF.

BSPR:

These results identify a new member of a complex pathway that, like other members of the pathway, is subject to regulation by inflammatory cytokines, and can therefore be used to modulate inflammatory reactions in which protein C or activated protein C is involved. Inhibition of the inflammatory response can be obtained by infusing soluble EPCR. Alternatively, localizing EPCR to surfaces in contact with blood will render the surfaces anticoagulant by virtue of the ability of EPCR to bind and concentrate the anticoagulant activated protein C at the surface. Alternatively, the function of EPCR can be enhanced by overexpressing the EPCR in endothelium that could be used to coat vascular grafts in patients with vascular disease or on stents in cardiac patients.

DRPR:

FIGS. 3A and 3B are flow cytometric analyses of F1-APC binding to 293T cells transfected with a cDNA clone of EPCR. Cells were transfected with a clone EPCR/pEF-BOS or pEF-BOS (negative control) by the calcium/phosphate method. After 24 h, cells were harvested and F1-APC binding was performed in the absence (dotted lines) or presence of 1.3 mM $CaCl_2$ (solid lines).

DRPR:

FIG. 4 is the predicted protein structure of EPCR based on nucleotide sequence, predicted amino acid sequence and a hydropathy plot of EPCR. The signal sequence and transmembrane region are indicated with the solid bars.

DRPR:

FIG. 5 is a comparison of the amino acid sequence of EPCR to the amino acid sequences of other

members of the CD1 family and CCD41. The EPCR sequence is shown in the first line and compared to murine CCD41 (second line), human CD1d (third line) and murine CD1.2 (fourth line). Identities with

EPCR are indicated by open boxes. Residues that are conserved between EPCR and all of the human CD1 family members are indicated by a double asterisk. Residues shared with one or more members of the CD1 family are indicated by a single asterisk.

DRPR:

FIG. 6 is a comparison of the amino acid sequence of human EPCR (first line) to the amino acid sequence of murine EPCR (second line). Identities are indicated by boxes. Similarities are indicated with an asterisk.

DEPR:

Human protein C and activated protein C are shown to bind to endothelium specifically, selectively and saturably ($K_d=30$ nM, 7000 sites per cell) in a Ca^{2+} dependent fashion. FL-APC binding to various human cell lines were examined, and found that the binding was HUVEC specific. A human kidney cell line transformed with SV40 large T antigen, 293T cells, expressed very few of these binding sites. A HUVEC cDNA library was constructed using the powerful mammalian expression vector, pEF-BOS (Mizushima and Nagata, (1990) *Nucleic Acids Res.* 18, 5322). Plasmid DNA was prepared from subpools of independent colonies (2,500 colonies per pool), and transfected into 293T cells, using the method of Kaisho et al., (1994) *Proc. Natl. Acad. Sci. (U.S.A.)* 91, 5325. FL-APC binding was analyzed on a flow cytometer. One of eight subpools gave a positive signal. This subpool was divided into 20 subpools and rescreened. After three rounds of enrichment, one positive clone, EPCR-1, was isolated. EPCR-1 carries a 1.3 kb insert. When transfected into 293T cells, this clone was capable of expressing the calcium-dependent binding site for FL-APC on the 293T cell surface.

DEPR:

Expression cloning revealed a 1.3 kb cDNA that coded for a type I transmembrane glycoprotein capable of binding protein C. This protein appears to be a member of the CD1/MHC superfamily. Like thrombomodulin, the receptor involved in protein C activation, the endothelial cell protein C receptor (EPCR) function and message are both down regulated by exposure of endothelium to TNF. Identification of EPCR as a member of the CD1/MHC superfamily provides insights into the role of this receptor for protein C in regulating the inflammatory response.

DEPR:

Total RNAs (15 μ g) from various cells were isolated, electrophoresed through formaldehyde agarose gels and transferred to a nylon membrane (Hybond-N.TM., Amersham). The 483 bp Xba I fragment from the 5' end of the EPCR cDNA was labeled by random priming according to the manufacturer's instructions (Multiprime.TM. DNA labeling system, Amersham) and used for hybridization.

DEPR:

Nucleotide and Predicted Protein Structure Analysis of EPCR

DEPR:

The cDNA is predicted to code for a protein of 238 amino acids (Sequence ID No. 2), which includes a 15 amino acid signal sequence (von Heijne, (1986) *Nucleic Acids Res.* 14, 4683-4690) at the N-terminal. Therefore, the mature protein is predicted to contain 223 amino acids. Sequence ID No. 2 is the predicted amino acid sequence of EPCR. Amino acids 1-15 of Sequence ID No. 2

(MLTTLLPILLSGWA) are the putative signal sequence determined by the method of von Heijne (von Heijne, 1986). Amino acids 211-236 of Sequence ID No. 2 (LVGLVLVGGFIIAGVAVGIFLCTGGR) are the putative transmembrane domain. Potential N-glycosylation sites are present at amino acids 47-49, 64-66, 136-138, and 172-174 of Sequence ID No. 2. Extracellular cysteine residues are present at amino acids 17, 114, 118, and 186 of Sequence ID No. 2. A potential transmembrane region (Engelman et al., (1986) *Annu. Rev. Biophys. Chem.* 15, 321-53) consisting of 23 amino acids was identified at the C-terminal end (beginning at amino acid 211 of Sequence ID No. 2).

DEPR:

DNA and protein database searches revealed that the sequence is related to the centrosome-associated, cell cycle dependent murine protein, CCD41, also referred as centrocyclin (Rothbarth et al., (1993) *J. Cell Sci.* 104, 19-30), as shown by FIG. 5. The similarity in the published sequence of murine CCD41 with human EPCR led to the cloning and sequencing of the murine EPCR. The sequence of murine EPCR is shown in FIG. 6. It is distinct from the published sequence of CCD41.

DEPR:

The EPCR amino acid sequence was also related to, but quite distinct from, the CD1/MHC superfamily and the murine CD1.2, as also shown by FIG. 5. Based on the homology to CD1/MHC, it is likely that EPCR contains two domains consisting of residues 17-114 and 118-188. Of the CD1 family members, CD1d is the most similar to EPCR. In the mouse, CCD41 is associated exclusively with the centrosome during G.sub.1 but becomes detectable elsewhere during the cell cycle, reaching a maximum during G.sub.2, except during the G.sub.2/M phase (Rothbarth et al., 1993). EPCR expression appears restricted to endothelium, which would not be expected for a cell cycle associated protein.

DEPR:

The identification of the protein C receptor on endothelium suggests that the endothelial cell binds protein C/APC through three distinct mechanisms. In addition to EPCR, protein S can bind APC/protein C on negatively charged membrane surfaces that include the endothelium (Stern et al., (1986) *J. Biol. Chem.* 261, 713-718), but this is not cell type specific (Dahlback et al., 1992). Thrombomodulin in complex with thrombin can bind protein C and APC (Hogg et al., 1992). On endothelium, the protein S binding sites (Nawroth and Stern, (1986) *J. Exp. Med.* 163, 740-745), thrombomodulin (Esmon, 1989) and EPCR are all down regulated by cytokines, indicating that inflammation can impair protein C pathway function at multiple levels.

DEPR:

The homology to the CD.sup.1/MHC family of proteins is especially interesting since it provides indications as to the function of EPCR. The CD1/MHC family has three extracellular domains termed .alpha.1, .alpha.2 and .alpha.3. The extracellular domain of EPCR contains four Cys residues that appear to correspond to two distinct domains. EPCR lacks the third domain of the CD1/MHC family, but the two domains have significant homology to the .alpha.1 and .alpha.2 domains of the CD1 protein family and the .alpha.2 domain of the MHC class I protein, suggesting that these proteins evolved from a common ancestor. The first domain of EPCR, residues 17-114, contains two potential N glycosylation sites and is rich in .beta. strand structure, suggesting that it may form a .beta. sheet. Despite the

.beta. strand structure, consensus sequences (Williams and Barclay, (1988) Ann. Rev. Immunol. 6, 381-405) for the immunoglobulin superfamily of receptors are absent. The second domain of EPCR, residues 118-188, contains two additional N glycosylation sites and, like the CD1/MHC family, this domain is predicted to have limited .beta. structure.

DEPR:

In vitro studies have suggested anti-inflammatory activities for APC. For instance, an unusual carbohydrate sequence on protein C can inhibit inflammatory cell adhesion to selectins (Grinnell et al., (1994) Glycobiology, 4, 221-226). Modest inhibitory effects of APC have been reported on TNF production (Grey et al., (1993) Transplant. Proc. 25, 2913-2914). EPCR could contribute to these anti-inflammatory mechanisms. Since the homologous protein family, CD1, can be linked to CD8 (Ledbetter et al., (1985) J. Immunol. 134, 4250-4254), it is also possible that the proteins C receptor is linked to another protein and signal through this second protein. One of the CD1 family members, CD1d, has been reported to promote T cell responses, possibly involving binding to CD8 (Panja et al., (1993) J. Exp. Med. 178, 1115-1119). CD1b has recently been reported to serve as an antigen presenting molecule (Porcelli et al., (1992) Nature 360, 593-597). The ability to bind protein C/APC could then be linked either directly or indirectly to signalling via direct interaction with cells of the immune system. Since the MHC class of proteins is involved in presentation of proteins to cell receptors, the concept of presentation of protein C/APC to inflammatory cells as a means of elaborating anti-inflammatory activity may also be involved. This includes modulation of enzyme specificity such as occurs with thrombin-thrombomodulin interaction (Esmon, 1989). In this case, the EPCR-APC complex might cleave biologically active peptides from unknown substrates.

DEPR:

EPCR mRNA Levels and APC Binding

DEPR:

To determine the cellular specificity of EPCR expression, the intensity of FL-APC binding to HUVEC was compared to several human cell lines. F1-APC bound strongly only to HUVEC, and not to any of the T, B, or monocytic cell lines tested. Cells were incubated at room temperature without or with 160 nM F1-APC in the presence of 1.3 mM CaCl₂. Binding was analyzed by flow cytometry. Slight binding was demonstrated with the osteosarcoma line, HOS and the epidermoid carcinoma cell line, HEp-2.

DEPR:

Total RNA was extracted from these cells and hybridized with the EPCR cDNA probe for Northern Blot Analysis. EPCR mRNA was detected by Northern blot analysis for HUVEC, Jurkat, HEp-2, Raji, HOS, and U937. Among the cells lines tested, EPCR mRNA was detected at high levels only in HUVEC. The calculated mRNA size of 1.3 kb was identical to the size of the isolated cDNA. After prolonged exposure, a weak signal was also detected with the osteosarcoma cell line HOS and monocyte cell line U937. Thus, both APC binding and EPCR mRNA expression are very specific for endothelium.

DEPR:

Effects of TNF on APC Binding and EPCR MRNA Levels

DEPR:

Several other members of the protein C anticoagulant pathway are subject

to regulation by

inflammatory cytokines (Esmon, 1989). For instance, endothelial cell surface thrombomodulin expression and message are known to be reduced by exposure of the cells to TNF (Conway and Rosenberg, 1988; Lentz et al., 1991). To determine if a similar process occurs with EPCR, HUVEC were treated with TNF and APC binding and expression of EPCR mRNA were examined. APC binding to HUVEC decreased in a time dependent fashion. EPCR activity decreased more rapidly than thrombomodulin antigen. HUVEC were cultured for 0, 6, 24 and 48 hr, in the presence of TNF- α (10 ng/ml). Cells were harvested and residual F1-APC binding or thrombomodulin (TM) expression was analyzed by flow cytometry. Cell surface TM was stained with an anti-TM murine monoclonal antibody and FITC-conjugated anti-mouse IgG. The negative control is without added fluorescent ligand.

DEPR:

HUVEC were treated with 10 ng/ml of TNF- α for 0, 0.5, 1, 2, 3, 6, 10 and 24 hr, and message was extracted and detected as described above. The results demonstrated that the concentration of EPCR mRNA was also reduced by TNF treatment. Message levels and APC binding activity decreased in parallel. Therefore, the TNF mediated down-regulation of APC binding to endothelium probably occurs at the level of mRNA expression.

DEPR:

Enhancement of inflammatory responses by blocking binding of endogenous molecules to EPCR can be achieved by administration of compounds binding to the receptor to a subject in need of inhibition. The degree of binding is routinely determined using assays such as those described above. Compounds which are effective include antibodies to the protein, fragments of antibodies retaining the binding regions, and peptide fragments of APC which include the Gla region. Inhibition of the inflammatory response could be obtained by infusing soluble EPCR. Alternatively, localizing EPCR to surfaces in contact with blood would render the surfaces anticoagulant by virtue of the ability of EPCR to bind and concentrate the anticoagulant APC at the surface. Alternatively, the function of EPCR could be enhanced by overexpressing the EPCR in endothelium used to coat vascular grafts in patients with vascular disease or on stents in cardiac patients.

DEPR:

Patients with thrombosis or hyperinflammatory conditions could be screened for defects in the EPCR gene. Sequence ID No. 1, and consecutive portions thereof of at least about seven nucleotides, more preferably fourteen to seventeen nucleotides, most preferably about twenty nucleotides, are useful in this screening using hybridization assays of patient samples, including blood and tissues. Screening can also be accomplished using antibodies, typically labelled with a fluorescent, radiolabelled, or enzymatic label, or by isolation of target cells and screening for binding activity, as described in the examples above. Typically, one would screen for expression on either a qualitative or quantitative basis, and for expression of functional receptor. Labelling can be with .sup.32 P, .sup.35 S, fluorescein, biotin, or other labels routinely used with methods known to those skilled in the art for labelling of proteins and/or nucleic acid sequences.

DEPR:

In cases where inflammatory mediators or vascular disease down regulate EPCR, it would be advantageous to increase its concentration in vivo on endothelium. The

binding assays described here
and the gene sequence allow assays for increased EPCR expression.
Similar approaches have been taken
with thrombomodulin and investigators have shown that cyclic AMP
(Maruyama, I. et al. (1991)
Thrombosis Research 61, 301-310) and interleukin 4 (Kapiotis, S. et al.,
(1991) Blood 78, 410-415)
can elevate thrombomodulin expression. The ability to screen such drugs or
drugs that block TNF down
regulation of EPCR provide an approach to elevating EPCR expression in
vivo and thus enhancing
anticoagulant and anti-inflammatory activity.

DEPR:

Studies based on inhibition of binding are predictive for indirect effects of
alteration of receptor
binding. For example, inhibition of binding of APC or increased expression
of TNF is predictive of
inhibition of EPCR function.

DEPR:

As described herein, a variety of compounds can be used to inhibit or
enhance expression of the
EPCR. The nature of the disorder will determine if the expression should
be enhanced or inhibited.

For example, based on the studies involving the use of an anti-protein C
antibody in combination
with cytokine, it should be possible to treat solid tumors by enhancing an
inflammatory response
involving blocking of protein C or activated protein C binding to an
endothelial cell protein
C/activated protein C receptor by administering to a patient in need of
treatment thereof an amount
of a compound blocking binding of protein C or activated protein C to the
receptor. Similarly, it
should be possible to treat disorders such as gram negative sepsis, stroke,
thrombosis, septic
shock, adult respiratory distress syndrome, and pulmonary emboli using a
method for inhibiting an
inflammatory response involving administration of EPCR or EPCR
fragments or substances that
upregulate EPCR expression to a patient in need of treatment thereof.

DEPL:

I. Cloning and Characterization of EPCR.

DEPL:

II. Modulation of Inflammation using EPCR.

8. Document ID: US 5804392 A

L1: Entry 8 of 24

File: USPT

Sep 8, 1998

US-PAT-NO: 5804392

DOCUMENT-IDENTIFIER: US 5804392 A

TITLE: Diagnostic assays using soluble endothelial cell protein C/activated
protein C receptor

DATE-ISSUED: September 8, 1998

INVENTOR-INFORMATION:

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US-CL-CURRENT: 435/7.1; 435/7.8, 435/975, 436/506, 530/387.1,
530/388.22, 530/389.1

AB: Plasma EPCR has been isolated, characterized and shown to
block cellular protein C
activation and APC anticoagulant activity. Plasma EPCR appears to be
about 43,000 daltons and
circulates at approximately 100 ng/ml (98.4.+-.27.8 ng/ml, n=22). Plasma
EPCR bound activated
protein C with an affinity similar to that of recombinant soluble EPCR
(Kd.sub.app
approximately 30 nM), and inhibits both protein C activation on an
endothelial cell line and
APC anticoagulant activity in a one-stage factor Xa clotting assay.
Soluble plasma EPCR appears
to attenuate the membrane-bound EPCR augmentation of protein C
activation and the anticoagulant
function of activated protein C. Soluble EPCR has also been detected in
urine. Levels of
soluble EPCR can rise in inflammatory disease associated with vascular
injury and appear to be
correlated with inflammation and disease states associated with abnormal
coagulation. Since
EPCR expression is restricted to larger vessels and is usually negative in
cappillaries, these
observations provide a mechanism for analyzing injury/stimulation of
large vessel endothelial
cells.

L1: Entry 8 of 24

File: USPT

Sep 8, 1998

DOCUMENT-IDENTIFIER: US 5804392 A

TITLE: Diagnostic assays using soluble endothelial cell protein C/activated
protein C receptor

ABPL:

Plasma EPCR has been isolated, characterized and shown to block cellular
protein C activation and
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approximately 100 ng/ml (98.4.+-.27.8 ng/ml, n=22). Plasma EPCR bound
activated protein C with an
affinity similar to that of recombinant soluble EPCR (Kd.sub.app
approximately 30 nM), and inhibits
both protein C activation on an endothelial cell line and APC anticoagulant
activity in a one-stage
factor Xa clotting assay. Soluble plasma EPCR appears to attenuate the
membrane-bound EPCR
augmentation of protein C activation and the anticoagulant function of
activated protein C. Soluble
EPCR has also been detected in urine. Levels of soluble EPCR can rise in
inflammatory disease
associated with vascular injury and appear to be correlated with
inflammation and disease states
associated with abnormal coagulation. Since EPCR expression is restricted
to larger vessels and is
usually negative in cappillaries, these observations provide a mechanism
for analyzing

injury/stimulation of large vessel endothelial cells.

BSPR:

Endothelial cells play a critical role in the protein C pathway in that they express two of the known receptors responsible for efficient protein C activation, thrombomodulin and the endothelial protein C/APC receptor (EPCR) (Fukudome and Esmon. 1994. *J. Biol. Chem.* 269:26486-26491; Stearns-Kurosawa, et al. 1996. *Proc. Natl. Acad. Sci. (USA)* 93:10212-10216). Thrombomodulin (CD141) is a transmembrane cofactor that binds circulating thrombin with high affinity and the resultant enzyme-cofactor complex is required for physiologically relevant protein C activation rates (Esmon and Owen. 1981. *Proc. Natl. Acad. Sci. (USA)* 78:2249-2252; Dittman, W. A. 1991. *Trends Cardiovasc. Med.* 1:331-336).

BSPR:

EPCR is a recently identified receptor with significant homology to the CD1/MHC class I family (Fukudome and Esmon, 1994; Fukudome, et al. 1996. *J. Biol. Chem.* 271:17491-17498; Regan, et al. 1996. *J. Biol. Chem.* 271:17499-17503). The cloning and biological role of the endothelial cell receptor for protein C was described in PCT/US95/09636 by Oklahoma Medical Research Foundation, entitled "Cloning and Regulation of an Endothelial Cell Protein C/Activated Protein C Receptor". The protein was predicted to consist of 238 amino acids, which includes a 15 amino acid signal sequence at the N-terminus, and a 23 amino acid transmembrane region which characterizes the receptor as a type I transmembrane protein.

BSPR:

EPCR binds both protein C and APC with similar affinity (Kd.sub.app about 30 nM) (Fukudome, et al., 1996) in the presence of calcium and facilitates protein C activation by presenting the protein C substrate to the thrombin-thrombomodulin activation complex on cell surfaces (Stearns-Kurosawa, et al., 1996). Both endothelial cell receptors are type I transmembrane proteins in which the ligand binds to an extracellular domain and both have a short intracellular cytoplasmic tail (Fukudome, et al. 1996; Jackman, et al. 1987. *Proc. Natl. Acad. Sci. (USA)* 84:6425-6429; Wen, et al., 1987. *Biochemistry* 26:4350-4357; Suzuki, et al. 1987. *EMBO J.* 6:1891-1897). In addition, their *in vitro* cell surface expression is down-regulated similarly by tumor necrosis factor- α (Fukudome and Esmon 1994). However, the characteristics of soluble forms of thrombomodulin and EPCR differ in several respects. Recombinant soluble thrombomodulin has reduced cofactor activity relative to the membrane form (Galvin, et al. 1987. *J. Biol. Chem.* 262:2199-2205; Parkinson, et al. 1990. *J. Biol. Chem.* 265:12602-12610). With both purified components and with cells, the changes in thrombin's substrate specifically induced by thrombomodulin result from competition for a shared binding domain on thrombin as well as conformational alterations in the active site pocket (Ye, et al. 1991. *J. Biol. Chem.* 266:23016-23021; Lu, et al. 1989. *J. Biol. Chem.* 264:12956-12962; Ye, et al. 1992. *J. Biol. Chem.* 267:11023-11028; Hofsteenge, et al. 1986. *Biochem. J.* 237:243-251; Mathews, 1994. *Biochemistry* 33:13547-13552; Esmon, et al. 1982. *J. Biol. Chem.* 257:7944-7947; Sadler, et al. 1993. *Haemostasis* 23:183-193). Soluble thrombomodulin also accelerates inactivation of thrombin by a variety of inhibitors (Bourin and Lindahl. 1993. *Biochem. J.* 289:313-330; Rezaie, 1995. *J. Biol. Chem.* 270:25336-25339). Both plasma and urine contain detectable thrombomodulin (Takano, et al. 1990. *Blood*. 76:2024-2029; Ishii and Majerus. 1985. *J. Clin. Invest.*

76:2178-2181) and because the thrombomodulin gene does not contain introns (Jackman, et al., 1987), these soluble forms are due to proteolysis of the extracellular domain at the cell surface.

BSPR:

In contrast, recombinant soluble EPCR (rsEPCR), truncated just before the transmembrane domain, binds both protein C and APC with an affinity similar to that observed for intact cell-surface expressed EPCR (Fukudome, et al. 1996). APC anticoagulant activity is inhibited effectively when bound to rsEPCR (Regan, et al., 1996), presumably because both rsEPCR and factor Va share binding determinants in a groove reminiscent of the anion binding exosite I in thrombin occupied by thrombomodulin (Mather, et al. 1996. *EMBO J.* 15:6822-6831). However, rsEPCR does not appear to influence proteolysis of small synthetic substrates by APC, nor inactivation of APC by α -1-antitrypsin or protein C inhibitor (Regan, et al., 1996). Unlike membrane-bound EPCR which enhances protein C activation (Stearns-Kurosawa, et al., 1996), rsEPCR has little effect on protein C activation by the soluble thrombin-thrombomodulin complex (Regan, et al., 1996), suggesting that any soluble forms of EPCR might inhibit protein C activation by competing with membrane-associated EPCR for protein C.

BSPR:

Immunohistochemistry indicates that EPCR is present primarily on the surface of endothelial cells from large vessels and is absent or present at low levels on most capillary endothelial cells.

BSPR:

It is therefore an object of the present invention to identify therapeutic and diagnostic uses for naturally occurring soluble EPCR.

BSPR:

It is a further object of the present invention to characterize naturally occurring soluble EPCR.

BSPR:

Plasma EPCR (has been isolated, characterized and shown to block cellular protein C activation and APC anticoagulant activity. Plasma EPCR appears to be about 43,000 daltons and circulates at approximately 100 ng/ml (98.4 \pm 27.8 ng/ml, n=22). Plasma EPCR was purified from human citrated plasma using ion-exchange, immunoaffinity, and protein C affinity chromatography. Flow cytometry experiments demonstrated that plasma EPCR bound activated protein C with an affinity similar to that previously determined from recombinant truncated EPCR (Kd.sub.app approximately 30 nM), defined as EPCR not including the transmembrane and cytoplasmic domains. Furthermore, plasma EPCR inhibited both protein C activation on an endothelial cell line and APC anticoagulant activity in a one-stage factor Xa clotting assay. Soluble EPCR has also been detected in human urine. Cloning of the gene encoding EPCR demonstrates that at least human EPCR can be alternatively spliced, yielding a truncated soluble EPCR including an insert unique to the alternatively spliced form (sEPCR). These results indicate that plasma EPCR can be derived either by proteolysis at the cell surface or by alternative splicing.

BSPR:

If the local concentrations of plasma EPCR are sufficiently high, particularly in disease states, the data indicates that the truncated soluble plasma EPCR could attenuate the membrane-bound EPCR augmentation of protein C activation and the anticoagulant function of

activated protein C. As

demonstrated by the examples comparing normal plasma EPCR with levels of EPCR from patients with autoimmune disease (systemic lupus erythematosus, SLE) and sepsis (a disorder involving both inflammation and coagulation abnormalities), levels of soluble EPCR appear to be correlated with

inflammation and disease states associated with abnormal coagulation. Assays are described based on measurement of soluble EPCR which are indicative of disease conditions involving coagulation, inflammation, and large vessel disease. Assay reagents are described, including isolated purified soluble EPCR, recombinant truncated soluble EPCR, and antibodies to the soluble EPCRs.

DRPR:

FIG. 1 is a schematic of the two known mechanisms for producing a soluble receptor as applied to EPCR, by proteolysis of the membrane-bound receptor to release an extracellular domain and leave the membrane anchor behind, and by alternative splicing of the mRNA, showing the sequences unique to membrane bound EPCR (mEPCR) and to proteolyzed plasma EPCR (pEPCR), and the sequence unique to soluble EPCR (sEPCR).

DRPR:

FIG. 3 shows the sequence inserted into human, bovine, murine, and baboon EPCR by alternative splicing.

DRPR:

FIG. 4a is a graph showing that soluble plasma EPCR binds to human protein C and APC. EA.hy926 cells were incubated with 60 nM fl-APC in the presence of 0-500 nM rsEPCR (circle-solid.) or plasma EPCR (largecircle.) for 30 minutes on ice. The cells were washed and cell-bound fluorescence was determined by flow cytometry as described. The intrinsic cell fluorescence in the absence of added fl-APC is indicated by the arrow. The mean cell fluorescence (MCF) plotted represents the average of duplicate MCF determinations.

DRPR:

FIGS. 4b and 4c are graphs showing soluble plasma EPCR and rsEPCR inhibit protein C activation on cell surfaces. In FIG. 4b, EA.hy926 cell monolayers were pre-incubated for 15 minutes at room temperature with 0.1 μ M protein C alone (quadrature.) or with 1 μ M rsEPCR (circle-solid.), or 2 μ g/ml 1494 mAb (largecircle.). Protein C activation was initiated by the addition of thrombin (2 nM final) and the reactions were stopped at the indicated times. Activated protein C was determined with an amidolytic assay and the activity rates in mOD/min are plotted for each time point. Control wells without added thrombin were included (box-solid.). Each data point represents the average of triplicate well determinations. In FIG. 4c, EA.hy926 cell monolayers were pre-incubated for 15 minutes at room temperature with 0.1 μ M protein C and the indicated concentrations of plasma EPCR (largecircle.) or rsEPCR (circle-solid.). Thrombin (final 2 nM) was added and the activation proceeded for 60 minutes at room temperature. The supernatants were added to a mixture of antithrombin and heparin and activated protein C activities (mOD/min) were determined with an amidolytic assay. Each data point represents the average of triplicate well determinations.

DRPR:

FIG. 4d is a graph showing soluble plasma EPCR inhibits APC anticoagulant activity. The anticoagulant activity of APC (25 nM) was determined with a one-stage Xa clotting

assay in the presence of 460 nM

plasma EPCR or rsEPCR. The effect was reversed when either soluble EPCR was pre-incubated for 5 minutes with 42 μ g/ml of 1496 mAb which blocks binding of APC to EPCR. The data represent the average of 4-6 determinations. \pm S.D.

DRPR:

FIG. 5 is a graph comparing levels of soluble plasma TM to soluble plasma EPCR in lupus patients, demonstrating that there is no correlation between TM and EPCR values, but that the majority of lupus patients exhibit extremely elevated levels of soluble plasma EPCR.

DEPR:

Endothelial Protein C Receptor, EPCR.

DEPR:

Previous investigations into the function of EPCR found that protein C binding to the membrane form of EPCR resulted in facilitation of protein C activation by the thrombin-thrombomodulin complex on cell surfaces (Stearns-Kurosawa, et al., 1996), but that soluble recombinant EPCR inhibited APC anticoagulant activity (Regan, et al. 1996). These observations, along with the knowledge that soluble thrombomodulin degradation products in plasma are a marker of endothelial damage in various disease states, led to the question of whether a soluble circulating form(s) of EPCR existed and, if so, what role it may have in the protein C pathway.

DEPR:

The examples demonstrate that a soluble form of EPCR circulates in plasma and is present in urine. In a healthy donor population, the plasma EPCR level was about 100 ng/ml and it appeared to be a single antigen species of approximately 43,000 daltons. Subsequent purification of the soluble EPCR from plasma and functional studies determined that it was capable of binding both protein C and APC with an affinity similar to intact membrane-bound EPCR. The in vitro studies using an endothelial cell line demonstrated that plasma EPCR inhibited protein C activation at near physiological concentrations of protein C and thrombin. In addition, direct addition of purified plasma EPCR to plasma resulted in inhibition of APC anticoagulant activity that was reversed with monoclonal antibodies to rsEPCR.

DEPR:

The identification of the purified plasma protein as being EPCR was based on comparison with the properties of rsEPCR. These proteins both reacted with the same battery of monoclonal and polyclonal antibodies, had the same amino-terminal sequence, bound to immobilized protein C in a Ca^{2+} -dependent fashion, and blocked protein C activation and APC anticoagulant activity with similar dose response curves. In addition, the affinities of both protein C and APC for rsEPCR and plasma EPCR are similar to the affinity of intact membrane-bound EPCR. These properties appear to be unique to EPCR.

DEPR:

Previous studies demonstrated that membrane-bound EPCR expressed on endothelial cells augments protein C activation by a factor of between three and five fold, whereas the examples demonstrate that the soluble form of EPCR purified from plasma inhibits protein C activation on endothelial cells and APC anticoagulant activity. This predicts that EPCR could modulate the protein C pathway in several ways. First, in the larger vessels where thrombomodulin concentration is low to the microcirculation, EPCR expression is correspondingly increased (Laszik,

et al., Circulation 1997).

Immunohistochemistry shows that in most organs, EPCR expression is most intense on large vessels and decreases progressively with decreasing vessel size, with little or no expression in the most abundant endothelial cell type, the capillary endothelium. EPCR expression may play a critical role in capturing the protein C substrate from the circulation and presenting it to the thrombin-thrombomodulin complex for activation. This is supported by in vitro observations that both the EA.hy926 endothelial cell line and human umbilical vein endothelial cells have at least six times more surface-expressed EPCR antigen than thrombomodulin. In the microcirculation where thrombomodulin concentration is high and EPCR is low, one would predict little influence on protein C activation. Finally, circulating soluble EPCR may reduce the generation of APC and the ability of APC to inactivate factor Va.

DEPR:

In a healthy individual, the soluble EPCR levels are about 2.5 nM, a concentration well below both the K_d of subunit 2 (approximately 30 nM) and the 80 nM protein C concentration in the circulation. Both of the effects of soluble plasma EPCR (inhibition of APC anticoagulant activity and protein C activation) required considerably higher concentrations than that present in normal plasma, leaving the question of the physiological role of the plasma EPCR uncertain. Patients with soluble EPCR levels that exceed 40 nM have been identified, as described in Example 3 (lupus). Thus, if the local concentration near the endothelial cell surface exceeds the systemic concentration, the soluble EPCR concentration would reach levels that would attenuate both APC generation and activity, contributing to thrombotic risk.

DEPR:

A soluble form of a receptor can be produced by proteolytic cleavage of the membrane-bound receptor or by alternative splicing mechanisms. Proteolysis at the membrane surface releases soluble thrombomodulin, and receptors for TNF, IL-1, IL-2, M-CSF, PDGF, and NGF (Heaney, et al. 1996. Blood 87:847-857). Soluble receptors have a multitude of potential functions including acting as antagonists of the membrane receptor, stabilizing the ligand, initiating ligand-mediated signaling, downmodulation of the membrane form, and binding to receptor inhibitors to indirectly facilitate receptor-ligand activity. The latter mechanism is used by the IL-1 receptor system in which the soluble isoforms of both IL-1 receptors are generated by proteolytic cleavage and tightly regulate the responsiveness to IL-1.alpha. and IL-1.beta. (Arend, et al. 1994. J. Immunol. 153:4766-4774).

The EPCR genomic structure contains an alternative splicing site which would code for a soluble protein truncated just before the transmembrane domain (Fukudome and Esmon. 1995. J. Biol. Chem. 270:5571-5577), as discussed below. Soluble IL-6 receptors appear to be generated by both proteolytic and alternative splicing mechanisms (Mullberg, et al. 1994. J. Immunol. 152:4958-4968; Lust, et al. 1992. Cytokine 4:96-100; Horiuchi, et al. 1994. Eur. J. Immunol. 24:1945-1948). This cleavage site can also be useful in recovering large quantities of soluble EPCR, by constructing an expression vector encoding the truncated EPCR immediately followed by a peptide sequence to which an antibody is specifically directed, as described in U.S. Pat. No. 5,298,599 to Morrissey and Esmon, the teachings of which are incorporated herein. The epitope will then be cleaved by proteolysis, before or after administration to a patient. See also U.S. Pat. No. 4,782,137

to Hopp et al.

DEPR:

Immunohistochemical studies have indicated that EPCR is located primarily on endothelium of large vessels and is barely detectable in capillaries. Plasma EPCR derived from membrane-bound EPCR, can therefore serve as a marker of large vessel disease processes. Plasma EPCR may serve as a useful comparison with plasma thrombomodulin levels which have been shown to be modulated in a variety of disease states, but which would reflect both large and small vessel disease processes, but probably would be dominated by small vessel contributions since most endothelium is microvascular.

DEPR:

The cDNA for EPCR is predicted to code for a protein of 238 amino acids (Sequence ID No. 2), which includes a 15 amino acid signal sequence (von Heijne, (1986) Nucleic Acids Res. 14, 4683-4690) at the N-terminal. Therefore, the mature protein is predicted to contain 223 amino acids. Direct sequencing of the recombinant protein showed that the mature protein started at Ser18. Sequence ID No. 2 is the predicted amino acid sequence of EPCR. Amino acids 1-15 of Sequence ID No. 2 (MLTLLPILLSGWA) are the putative signal sequence determined by the method of von Heijne (von Heijne, 1986). Amino acids 211-236 of Sequence ID No. 2 (LVLGVLVGGFHAGVAVGIFLCTGGR) are the putative transmembrane domain. Potential N-glycosylation sites are present at amino acids 47-49, 64-66, 136-138, and 172-174 of Sequence ID No. 2. Extracellular cysteine residues are present at amino acids 17 (removed in plasma EPCR), 114, 118, and 186 of Sequence ID No. 2. A potential transmembrane region (Engelman et al., (1986) Annu. Rev. Biophys. Chem. 15, 321-53) consisting of 23 amino acids was identified at the C-terminal end (beginning at amino acid 211 of Sequence ID No. 2).

DEPR:

The protein is a type 1 transmembrane protein. The extracellular domain contains four potential N-glycosylation sites and three Cys residues. Glycosylation is not essential for activity, as shown by N-glycanase digestion. The cytoplasmic region contains only three amino acids and terminates with a Cys, which is palmitoylated. If the terminal cysteine is not properly palmitoylated, the protein may be secreted. Altering the sequence of the EPCR to replace this cysteine with another amino acid thereby provides a means for making an essentially full length EPCR which is secreted instead of being membrane bound.

DEPR:

Details of the following studies and results are described in the examples. Human plasma contains about 100 ng/ml of soluble EPCR (Table 1). This was measured by an enzyme linked immunoassay (ELISA) using two monoclonal antibodies (1494 mAb and 1495 mAb) and standard techniques. Significantly elevated soluble EPCR levels were found in patients with systemic lupus erythematosus and sepsis. These levels seemed fairly high for a membrane-bound receptor that is present, with few exceptions, only on the surface of the large blood vessels. To put this in perspective, thrombomodulin (TM) is expressed on all endothelium, as well as some non-vascular cells, yet normal soluble TM levels are only about 10-40 ng/ml (Takano, et al., Blood 76:2024-2029, 1990). The soluble TM levels were elevated in the patients with lupus, but not sepsis. Importantly, there was no correlation between the plasma EPCR and TM levels in these patient groups (r.s.p.2 = 0.028

and 0.034, respectively).

DEPR:

The lack of correlation between the plasma EPCR and TM levels and the high plasma EPCR concentration is consistent with the concept that plasma EPCR originates from both proteolytic and alternative splicing mechanisms. The genomic structure of human EPCR contains four exons, separated by introns.

Review of this sequence reveals an in-frame reading sequence after the exon III-intron III boundary (at the 5' GT) that includes a TAA stop codon at position 7527. Since this stop codon is upstream of exon IV that codes for the transmembrane domain, the predicted protein would contain a unique 48 residue carboxyl-terminal tail (coded for by the intron sequence) and would not contain a transmembrane anchor.

DEPR:

FIG. 1 is a diagram of two potential ways truncated EPCR can be derived: by proteolysis immediately before the transmembrane domain or by alternative splicing. As shown by FIG. 2, alternative splicing results in inclusion of a peptide sequence in the alternatively spliced truncated EPCR. As shown by

FIG. 3, this sequence is highly conserved between species, although slight differences exist, resulting in a new carboxyl-terminal tail of 48 residues for human and bovine EPCR, 51 residues for murine EPCR, and 22 residues for baboon EPCR.

DEPR:

Patient samples can be screened for the presence of, and amount of, sEPCR or EPCR, using antibodies to either EPCR, the unique insert present in the alternatively spliced insert in EPCR, or antibodies which bind with greater affinity to either EPCR or sEPCR due to conformational differences. Samples can also be screened using other standard techniques to specifically quantitate proteins which are present.

DEPR:

Antibodies to EPCR, and in particular, soluble EPCR ("sEPCR"), and recombinant soluble EPCR ("rsEPCR") can be generated which are useful in detection, characterization or isolation of receptor proteins, as well as for modifying receptor protein activity, in most cases, through inhibition of ligand binding. Antibodies are generated by standard techniques, using human or animal purified or recombinant receptor proteins or fragments thereof as the immunogen.

DEPR:

Monoclonal antibodies to EPCR were obtained as described for other proteins by Esmon, et al., 1993.

Methods Enzymol. 222:359-385. The antibodies referred to as 1494, 1495, and 1496 mAbs are IgG1.sub.K

antibodies that bind to recombinant soluble EPCR and to cell surface-expressed EPCR. The 1494 and

1496 mAbs block the binding of protein C and APC to EPCR, and inhibit the ability of cellular EPCR

to facilitate protein C activation by the thrombin-thrombomodulin complex. The 1495 mAb does not

block ligand binding to EPCR, does not alter cell surface protein C activation, and has a binding epitope distinct from that for 1494 or 1496 mAb. The antibodies can be labelled using standard

techniques, such as radiolabelling, enzyme labelling, fluorescent labels such as fluorescein, gold

particles, dyes, and other means for detection of the antibodies. For example, antibody can be

biotinylated with biotinamidocaproate N-hydroxysuccinimide ester using standard procedures. Antibody

can be immobilized to a solid support for use in immunoassays, for example, AffiGel-10.TM.,

nitrocellulose, or microtiter wells, or use in solution phase immunoassays.

DEPR:

In a preferred embodiment, EPCR is measured using microtiter plates (Maxisorp.TM., NUNC NS,

Roskilde, Denmark) coated with 50 microliters of 4 micrograms/ml 1495 mAb in 15 mM Na.sub.2

CO.sub.3, 35 mM NaHCO.sub.3, pH 9.6, at 4.degree. C. overnight. At room temperature, the plates are

then washed three times with 20 mM Tris-HCl, 0.1M NaCl, 0.05% Tween 20, pH 7.5 (assay buffer), and

blocked with assay buffer containing 0.1% (wt/vol) gelatin for at least one hour. The wells are then

washed, 50 microliter samples added in triplicate wells, and the plates incubated for one hour. The

wells are aspirated, washed three times with assay buffer, and 50 microliters of 2 micrograms/ml

biotin-1494 mAb added. The plates are incubated for 1 hour, washed three times, and 50 microliters

of 0.25 micrograms/ml streptavidin-alkaline phosphatase conjugate (GIBCO BRL) added and incubated

for an additional hour. The wells are washed five times, and the substrate and amplifier reagents

from an ELISA amplification kit (GIBCO BRL) added sequentially at 15-min intervals according to the

manufacturer's directions. The color development is stopped with 0.3M H.sub.2 SO.sub.4, and the

endpoint absorbance read at 490 nm on a V.sub.max microplate reader. Standards in triplicate wells

are from 1.5 to 100 ng rsEPCR/ml in 20 mM Tris-HCl, 0.1M NaCl, and 1 mM EDTA, 0.1% gelatin, pH 7.5.

The standard curve is linear from 1.5 to 12.5 ng/ml, and samples are diluted with the same buffer to

fall within the linear range. Studies show that between one and two percent plasma does not affect

the linearity of the assay or the sensitivity of the standard curve. Plasma samples from healthy

volunteers were diluted with assay buffer containing 1 mM EDTA to a final 2% plasma, and EPCR

antigen levels are calculated from the average of triplicate wells by reference to standard curve

determined on the same plate.

DEPR:

The assay for soluble EPCR is useful in detection and analysis of coagulation and inflammatory

states and disorders as discussed herein, such as autoimmune diseases like lupus, in transplant

monitoring, sepsis, shock, pre-eclampsia, diabetes, cardiopulmonary bypass, unstable angina,

restenosis, angioplasty (i.e., vascular disease), kidney or liver disease. For example, the EPCR is

a marker for large blood vessels, and therefore for damage to large blood vessels. An increase in

the amount of soluble EPCR is indicative of large vessel injury, resulting either in proteolysis of

EPCR or stimulation of sEPCR synthesis. The ratio of EPCR to thrombomodulin can also be determined,

based on either blood or urine samples, which is indicative of the relative extent of microvascular

versus large vessel. The relative amounts of EPCR to cytokines, leukocyte activation markers and

complement factors or activation markers can also be used to indicate disease state.

DEPR:

Since EPCR is present on endothelial cells, it is useful as a marker of endothelial cell damage. It

can be used as an indicator of drug effect, both toxicity as well as efficacy. For example, in lupus

patients, drugs effectively minimizing inflammatory/coagulation mediated, large vessel injury would

result in decreasing EPCR levels.

DEPR:

The following abbreviations are used: rsEPCR, recombinant soluble EPCR with the HPC4 epitope

inserted in place of the transmembrane domain and cytosolic tail; mAb, monoclonal antibody;

SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis.

DEPR:

Proteins. Human protein C (Esmon, et al. 1993. *Methods Enzymol.* 222:359-385), bovine thrombin (Owen, et al. 1974. *J. Biol. Chem.* 249:594-605), and bovine antithrombin (Esmon 1977. "Factors regulating the inhibition of thrombin by antithrombin III. In *Chemistry and Biology of Thrombin*". R. L. Lundblad, J. W. Fenton, II, and K. G. Mann, editors. Ann Arbor Science, Ann Arbor. 403-411) were purified as described. Recombinant soluble EPCR, rsEPCR, consists of the extracellular domain of EPCR truncated at residue 210 just before the transmembrane domain, followed by a 12 residue sequence that permits calcium-dependent immunoaffinity purification on the HPC4 monoclonal antibody (Takahashi, et al. 1992; Stearns, et al. 1988. *J. Biol. Chem.* 263:826-832). The construction, purification, and protein C/APC binding characteristics of rsEPCR (Fukudome, et al. 1996). Goat preimmune serum and polyclonal antiserum to rsEPCR was prepared and the IgG purified (Fukudome, et al. 1996). Goat anti-rsEPCR polyclonal antibody was biotinylated with biotinamidocaproate N-hydroxysuccinimide ester using standard procedures.

DEPR:

Monoclonal antibodies. Monoclonal antibodies (mAb) against rsEPCR were obtained as described for other proteins (Esmon, et al. 1993). The 1494, 1495, and 1496 mAb are IgG1k antibodies that bind to rsEPCR and to cell surface-expressed EPCR. The 1494 and 1496 mAb block the binding of protein C and APC to EPCR and inhibit the ability of cellular EPCR to facilitate protein C activation by the thrombin-thrombomodulin complex (Stearns-Kurosawa, et al. 1996). The 1495 mAb does not block ligand binding to EPCR, does not alter cell surface protein C activation and has a binding epitope distinct from that for 1494 or 1496 mAb. The 1494 and 1495 mAbs were biotinylated with biotinamidocaproate N-hydroxysuccinimide ester using standard procedures. The 1494 mAb was coupled to AffiGel-10, according to the manufacturer's directions, for immunoaffinity purification of plasma EPCR. The screening of anti-EPCR mAb was done using methods described by Stearns-Kurosawa, et al. (1996); Fukudome, et al. (1996).

DEPR:

Clotting Assay. The effect of rsEPCR or purified plasma EPCR on APC (25 nM) anticoagulant activity in a one-stage factor Xa clotting assay was performed (Regan, et al. 1996) in the presence or absence of 83 .mu.g/ml 1496 mAb, an antibody that blocks APC-EPCR interaction (Stearns-Kurosawa, et al. 1996). The soluble EPCRs and 1496 mAb were pre-incubated for 15 minutes before assay.

DEPR:

Flow Cytometric Analysis. To serve as a fluorescent probe, APC was labeled with fluorescein in the active site (fl-APC) as described (Fukudome and Esmon, 1994; Bock, P. E. 1988. *Biochemistry* 27:6633-6639). The effect of rsEPCR or plasma EPCR on APC binding to EA.hy926 cells was studied by flow cytometry (Fukudome, et al. 1996). Briefly, harvested cells were incubated for 30 min on ice with 60 nM fl-APC in the absence or presence of increasing concentrations of either soluble EPCR preparation, washed, and cell-bound fluorescence was determined by flow cytometry with 10,000 events counted per sample. All assays were done in Hank's balanced salt solution supplemented with 1% bovine serum albumin, 3 mM CaCl₂.sub.2, 0.6 mM MgCl₂.sub.2, and 0.02% sodium azide.

DEPR:

Cell surface protein C activation. EA.hy926 cells were cultured in 96-well

tissue culture dishes

(Stearns-Kurosawa, et al. 1996). The confluent monolayers were washed three times with Hank's balanced salt solution supplemented with 1% (w/v) bovine serum albumin, 3 mM CaCl₂.sub.2, 0.6 mM

MgCl₂.sub.2, and 0.02% sodium azide. All assays were done at room temperature in the same buffer in 60 .mu.l final volume, and all protein concentrations represent the final concentration in the

assay. Protein C was added (0.1 .mu.M) in the absence or presence of rsEPCR, plasma EPCR, or 1494 mAb at the indicated concentrations and pre-incubated with the cells for 15 minutes. Thrombin was added to the mixtures (2 nM) to start the activation reactions. At the indicated time, 50 .mu.l aliquots were removed and added to 10 .mu.l of antithrombin (0.7 .mu.M final) and heparin (5 U/ml final) in a 96-well microtiter plate. APC amidolytic activity was determined by addition of

Spectrozyme PCa substrate (0.2 mM) and the rate of change in absorbance at 405 nm (mOD/min) was measured on a Vmax kinetic microplate reader (Molecular Devices, Menlo Park, Calif.). All assay points were done in triplicate wells and less than 10% of the protein C substrate was activated as determined by reference to a standard curve of fully activated protein C versus mOD/min.

DEPR:

ELISA for quantitation of plasma EPCR. An enzyme-linked immunosorbent assay for detection of EPCR antigen in plasma was developed. Microtitre plates (Maxisorp; Nunc, Roskilde, Denmark) were coated with 50 .mu.l of 4 .mu.g/ml 1495 mAb in 15 mM Na₂CO₃.sub.3, 35 mM NaHCO₃.sub.3, pH 9.6 at 4.degree. C. overnight. The following steps were done at room temperature. The wells were washed three times with 20 mM Tris-HCl, 0.1M NaCl, 0.05% Tween 20, pH 7.5 (assay buffer) and blocked with assay buffer containing 0.1% (w/v) gelatin for at least one hour. The wells were washed, 50 .mu.l samples were added in triplicate wells, and the plates were incubated for one hour. The wells were aspirated, washed three times with assay buffer and 50 .mu.l of 2 .mu.g/ml biotin-1494 mAb was added. The plates were incubated for one hour, washed three times and 50 .mu.l of 0.25 .mu.g/ml streptavidin-alkaline phosphatase conjugate (GibcoBRL) was added and incubated for an additional one hour. The wells were washed five times and the substrate and amplifier reagents from an ELISA amplification kit (GibcoBRL) were added sequentially at 15 minute intervals according to the manufacturer's directions. The color development was stopped with 0.3M H₂SO₄.sub.2 SO₄.sub.4 and the endpoint absorbance at 490 nm was read on a Vmax microplate reader. Each plate contained standards in triplicate wells from 1.5-100 ng/ml rsEPCR in 20 mM Tris-HCl, 0.1M NaCl, 1 mM EDTA, 0.1% gelatin, pH 7.5. The standard curve was linear (r=0.99) from 1.5-12.5 ng/ml and plasma samples were diluted with the same buffer to fall within the linear range. Preliminary experiments determined that a final concentration of 1-2% human plasma did not affect the linearity or sensitivity of the standard curve. Plasma samples from healthy volunteers were diluted with assay buffer containing 1 mM EDTA to a final 2% plasma and EPCR L, antigen levels were calculated from the average of triplicate wells by reference to a standard curve determined on the same plate.

DEPR:

An alternative assay was developed in which the coating and detecting antibodies were reversed (1494 mAb coating; biotin-1495 mAb detecting) and antibody binding was detected with the Blue Phos substrate (KPL Laboratories; Gaithersburg, Md.). this method was used to assay plasma EPCR in the

sepsis patients. This assay was more sensitive, probably because of affinity differences, but both assays gave qualitatively similar results.

DEPR:

Purification of plasma EPCR. Plasma EPCR as purified from human citrated plasma (Oklahoma Blood Institute) using a combination of ion-exchange chromatography, anti-rsEPCR mAb immunoaffinity chromatography, and chromatography on protein C affinity columns. Two preparations were done in slightly different ways.

DEPR:

In the first preparation, plasma (1L) was diluted with an equal volume of 20 mM Tris-HCl, pH 7.5, 10 mM benzamidine, 400 units sodium heparin and batch-adsorbed for 1 hour with 1 g pre-swollen QAE resin. After settling, the resin was processed for purification of protein C (Esmon, et al. 1993).

Solid ammonium sulfate was added to the supernatant at 4.degree. C. to 40% saturation, centrifuged, and additional ammonium sulfate was added to that supernatant to achieve 70% saturation. After

centrifugation, the soft pellet was placed in dialysis bags and dialyzed overnight against 12 L of

20 mM Tris-HCl, 0.02% sodium azide, pH 7.4. The dialysate was applied to a 1496 mAb-AffiGel-10 immunoaffinity column (6 ml resin; 5 mg IgG/ml resin) equilibrated in 20 mM Tris-HCl, 0.1M NaCl,

0.02% sodium azide, pH 7.4. The column was washed with more than 12 ml of the same buffer and eluted with 50% (v/v) ethylene glycol in 20 mM Tris-HCl, pH 7.4 (Jun Xu, unpublished observations). The

peak fractions from the elution were pooled (0.37 total OD.sub.280), concentrated (Centriprep 30,

Millipore), and the buffer exchanged to 20 mM Tris-HCl, 0.1M NaCl, 3 mM CaCl.sub.2, 0.6 mM

MgCl.sub.2, 0.02% sodium azide, pH 7.4. This material was applied to a protein C affinity column

that had been previously prepared by applying the purified protein C (3 mg) to an HPC4-AffiGel-10 column (5 mg IgG/ml resin; 0.9.times.8 cm) in the same buffer. The HPC4 mAb binds the protein C

activation region in a calcium-dependent fashion (Esmon, et al. 1993; Stearns, et al. 1988) and does

not interfere with subsequent binding of EPCR to protein C. After applying the sample containing plasma EPCR, the column was washed with approximately 12 ml of buffer and eluted with 20 mM

Tris-HCl, 0.1M NaCl 5 mM EDTA, 10 mM MOPS, 0.02% sodium azide, pH 7.5. Fractions were monitored for

absorbance at 280 nm and for EPCR antigen using the ELISA described above. The eluate containing

both protein C and plasma EPCR was applied to an FPLC (Pharmacia-LKB, Uppsala, Sweden) Mono Q column

and the column developed with a linear gradient of 0.1-1M NaCl in 20 mM Tris-HCl, pH 7.5. About

half of the plasma EPCR did not bind to the Mono Q column, half eluted at about 0.2M NaCl, and the

protein C eluted at approximately 0.5M NaCl. Both ionic species of plasma EPCR appeared identical on

SDS-PAGE gels under reducing or non-reducing conditions with silver staining, with Coomassie BB

staining or with gold staining (Pierce) after transfer to PVDF membranes, and on Western blots with

the biotin-polyclonal anti-rsEPCR antibody probe.

DEPR:

The second preparation of plasma EPCR was done starting with 4L of plasma to purify enough protein for functional studies. In this case, the 1496-AffiGel-10 resin (20 ml of 5 mg IgG/ml resin) was

added directly to the citrated plasma, along with final concentrations of 10 mM benzamidine, 1 mM

diisopropylfluorophosphate, and 0.5 units/ml sodium heparin. The plasma was batch-adsorbed overnight

at 4.degree. C. with gentle mixing. After the resin settled, the supernatant

was processed for

protein C purification (Esmon, et al. 1993). The resin was packed into a 2.5.times.30 cm column,

washed extensively with 20 mM Tris-HCl, 0.1M NaCl, 0.02% sodium azide, pH 7.4 and eluted with 50%

ethylene glycol in 20 mM Tris-HCl, pH 7.4. The eluate was pooled and concentrated (5.5 total

OD.sub.280), applied to a Mono Q column and the two ionic species (breakthrough and 0.2M NaCl eluate

peak) were re-applied to the 1496-AffiGel-10 resin (1.5.times.11 cm). The column was eluted with 50%

ethylene glycol as before. The eluate (0.71 ODs) was concentrated and the buffer exchanged to 20 mM

Tris-HCl, 0.1M NaCl, 3 mM CaCl.sub.2, 0.6 mM MgCl.sub.2, 0.02% sodium azide with a Centriprep 30.

This material was then applied to an affinity column in which protein C (2.9 mg) had been initially

applied in the same buffer to an HPC2-AffiGel-10 column (0.6.times.17 cm). The HPC2 mAb binds to the

protein C serine protease domain and does not interfere with EPCR binding (Fukudome, et al. 1996).

The bound EPCR was eluted with buffer containing 5 mM EDTA.

Contaminating serum amyloid P (from the protein C sample) was removed by ion-exchange chromatography on the

FPLC Mono Q column. The sample was applied in 0.2M NaCl, so that the plasma EPCR did not bind, and was

separated from the contaminants which eluted at 0.4-0.5M NaCl. The resultant purified

plasma EPCR (0.193 OD.sub.280)

appeared homogenous by SDS-PAGE with silver staining and by Western blotting with polyclonal

anti-rsEPCR. This material was used for the functional studies and amino-terminal sequence analysis.

DEPR:

Protein Sequencing. The amino-terminal sequence analysis of soluble plasma EPCR was performed in Dr.

Kenneth Jackson's laboratory at the Molecular Biology Research Facility, W. K. Warren Medical

Research Institute, Oklahoma City. Amino acids are designated by the standard one letter code.

DEPR:

Plasma EPCR purity was determined from silver stained SDS-PAGE 10% gels and western blots of

membranes probed with biotin-goat anti-rsEPCR (reduced and non-reduced). A single band of

approximately 43,000 Da appears in both the serum and plasma samples after the membrane is probed

with the polyclonal antibody. The size of the protein detected appears slightly larger than the

rsEPCR. The other bands detected were background binding of IgG as judged by probing with preimmune

IgG and longer exposure times. overnight incubation of plasma samples with the anti-EPCR 1495 mAb

coupled to AffiGel-10 resin, followed by washing and elution of bound antigen under reducing

conditions, resulted in a single band detected by Western blotting with biotin-goat anti-rsEPCR

polyclonal antibody.

DEPR:

Determination of soluble EPCR antigen in plasma from healthy volunteers by ELISA using mAb 1495 as

the coating antibody found antigen levels of 91.1 +/- 24.5 ng/ml in females (n=12) and 107.2 +/-

30.2 ng/ml in males (n=10). When calculated together, the average plasma EPCR antigen level was 98.4

+/- 27.8 ng/ml. The value for males appeared to be slightly higher than for females, similar to

thrombomodulin (Quehenberger, et al. Thromb. Haemost. 76: 729-734), although the population studied

was too limited for statistical analysis and this study was not designed to assess differences due

to gender, age, diet or other variables.

DEPR:

Since the plasma EPCR appeared to be a single species at approximately 100 ng/ml, it became important to determine whether the circulating EPCR could bind protein C and APC. Soluble EPCR was purified from human plasma by a combination of ion-exchange chromatography, precipitation with ammonium sulfate, and immunoadsorption by anti-EPCR 1496 mAb-AffiGel-10 column chromatography as described in Experimental Procedures.

DEPR:

This plasma EPCR (approximately 110 .mu.g) was applied to a protein C affinity column prepared by applying protein C (3 mg) to an anti-protein C HPC4 mAb-AffiGel-10 column in buffer containing 3 mM CaCl₂.sub.2, 0.6 mM MgCl₂.sub.2. The column was washed and plasma EPCR was applied at fraction 19. The column was washed and eluted with buffer containing 5 mM EDTA starting at fraction 35. Absorbance at 280 nm and EPCR antigen was determined for the fractions. EPCR antigen was determined by ELISA.

DEPR:

More than 98% of the applied plasma EPCR antigen bound to the protein C affinity column. The absorbance profile indicates co-elution of EPCR and protein C from the antibody column, consistent with the calcium-dependence of protein C binding to this antibody (Stearns, et al. 1988).

DEPR:

To purify sufficient protein for functional and structural studies, EPCR was purified from 4L of plasma using a similar, but slightly modified procedure. After elution from a protein C-antibody affinity column, residual contaminating proteins were removed by ion-exchange chromatography on an FPLC Mono Q column. The resultant preparation of plasma EPCR appeared homogenous on SDS-PAGE 10% gels with silver staining and identical results were obtained with western blots probed with biotin-goat anti-rsEPCR polyclonal antibody under both reducing and non-reducing conditions. Amino-terminal sequence analysis of the purified protein yielded only one sequence, S-Q-D-A-S-D, which is identical to the amino-terminal sequence of recombinant soluble EPCR (Sequence ID No. 2). This is the first amino-terminal sequence determination of EPCR from a natural source.

DEPR:

The ability of plasma EPCR to bind to APC was assessed by competition studies in which plasma EPCR was allowed to compete with cellular EPCR for APC, and the resultant free APC that could bind to cellular EPCR was assessed by flow cytometry (FIG. 4a). APC labeled with fluorescein in the active site (fl-APC) was incubated with EA.hy926 cells in the presence or absence of either plasma EPCR or rsEPCR. The EPCR concentration dependence for inhibition of APC binding to the cells was similar for both soluble forms of EPCR. This observation indicates that the affinity of plasma EPCR for binding APC is similar to that previously determined for the rsEPCR-APC binding interaction (K_d.sub.app approximately 30 nM).

DEPR:

While rsEPCR has little effect on protein C activation in a soluble system (Regan, et al. 1996), membrane-bound EPCR has a very potent ability to facilitate activation on cell surfaces (Stearns-Kurosawa, et al. 1996). The current data demonstrating the existence of a circulating form of EPCR capable of binding protein C and APC suggested that plasma EPCR has the potential to alter cell-surface activation of protein C. The thrombin-dependent activation of an approximately

physiological level of protein C (0.1 .mu.M) on EA.hy926 cells was inhibited by excess rsEPCR almost to the level of that observed with the anti-rsEPCR 1494 mAb that blocks the EPCR-protein C binding interaction, as shown by FIG. 4b. Previous studies have demonstrated that rsEPCR has no effect on APC amidolytic activity using small synthetic substrates (Regan, et al. 1996). The plasma EPCR was slightly more effective in its ability to inhibit cell-surface protein C activation on the EA.hy926 cells relative to the rsEPCR, as shown by FIG. 4c.

DEPR:

In a one-stage factor Xa clotting assay, purified plasma and soluble recombinant EPCR inhibited the APC prolongation of clotting times similarly (FIG. 4d). Inhibition of APC anticoagulant activity by rsEPCR had been observed previously (Regan, et al. 1996). As expected, the 1496 mAb reversed this effect by blocking the APC-plasma EPCR binding interaction.

DEPR:

To address the question of whether soluble EPCR is present in urine, four urine samples were collected (first morning void) and analyzed for the presence of soluble EPCR by western blotting and ELISA.

DEPR:

Undiluted pediatric urine samples were compared to a 4% normal plasma and recombinant soluble EPCR (1 ng). The samples were incubated with biotin-goat-anti-rsEPCR and a streptavidin-alkaline phosphatase detection system.

DEPR:

The western blot indicates that a) soluble EPCR is present in urine, and b) the soluble EPCR antigen is present at a size similar to that observed in plasma. Obvious degradation is not observed. The amount of soluble EPCR in the four samples as quantified by ELISA was 40.3, 6.1, 35.6, and 90.1 ng/ml.

DEPR:

Normal human plasma EPCR concentration are about 100 ng/ml (98.4 +/- 27.8 ng/ml; 2.5 nM), as discussed above. A panel of samples from patients with lupus erythematosus (n=54) was assayed and soluble EPCR levels were found to range from non-detectable levels to greater than 1,700 ng/ml. Fifteen patients had soluble EPCR levels greater than 200 ng/ml.

DEPR:

Previous studies have shown elevated soluble plasma TM levels in lupus patients due to endothelial damage and the current lupus patient samples were assayed for plasma TM as a reference. It was found that their soluble TM levels had absolutely no correlation with their soluble EPCR levels, as shown by FIG. 5. This is an important observation that suggests that the source of the soluble plasma EPCR is not simply from randomly damaged endothelium. In contrast to TM, membrane-bound EPCR expression in humans and primates is restricted primarily to the endothelium of large vessels, with capillaries expressing little EPCR. The distinctive localization of EPCR is expected to augment protein C activation locally to prevent large vessel thrombosis. The primary localization of membrane-bound EPCR to the large vessels points to a targeted thrombotic risk in the large vessels that may be predicted by soluble plasma EPCR concentrations.

DEPR:

Patient blood samples were taken at time 0 (entry into the Intensive Care Unit, ICU) and at two days and six days after treatment with anti-thrombin II (ATII) or a placebo.

Plasma soluble EPCR and soluble thrombomodulin (TM) were assayed only on time 0 samples.

DEPR:

These results are shown graphically in FIG. 6. As in the lupus patients, patients with sepsis show very significant elevations in plasma EPCR levels, not correlated with soluble TM levels.

DEPR:

The observation that soluble plasma EPCR inhibits both protein C activation and activated protein C anticoagulant activity indicates that the elevated plasma EPCR levels in these patients poses an additional thrombotic risk and marks evidence of vascular injury/responsiveness. Examples of conditions these are indicative of include disorders associated with endothelial cell stimulation, atherogenesis, leukocyte adhesion and plaque rupture.

DEPR:

As an initial approach to determine whether a soluble EPCR isoform could be generated by alternative splicing mechanism, RNA was isolated from human and baboon tissues and reverse transcriptase-PCR (RT-PCR) performed with gene-specific primers. Although the baboon EPCR genomic sequence is not known, primers based on the human sequence were used based on the reasoning that baboons and humans are closely related on the evolutionary scale.

DEPR:

In the RT-PCR procedure generally, total RNA is isolated from homogenized tissue. The RNA is mixed with a specific antisense primer, nucleotides and the reverse transcriptase enzyme. In the mix, the RNA serves as a template for the reverse transcriptase to create a first strand cDNA. This new cDNA template is then amplified by conventional PCR using specific primers and Taq polymerase. Primers that would amplify both the membrane form of EPCR (424 bp) and the predicted alternatively spliced product (674 bp) were chosen. Products corresponding to both forms of EPCR were amplified from a variety of baboon tissues (FIG. 4) and human lung and placenta. Possible contamination with genomic DNA was unlikely as judged by controls without reverse transcriptase and the lack of a 1,885 bp band in the reactions with the tissues.

DEPR:

A. EPCR ELISA: The coating antibody is 1494 mAb that binds to the ligand binding domain of EPCR. The detecting antibody is biotinylated 1495 mAb, which does not block protein C/APC binding, and does not cross-react with 1494 mAb. The detection system is streptavidin-alkaline phosphatase and BluePhos substrate (from KPL).

DEPR:

The observation that the predicted soluble EPCR isoforms will have unique carboxyl terminal tails provides a structural difference for distinguishing between the isoforms using isoform-specific antibodies. The working model is that plasma levels of proteolyzed soluble EPCR will report endothelial injury, whereas levels of alternatively spliced soluble EPCR will report an endothelial response to stimuli. It is anticipated that the relative plasma levels of the soluble EPCR isoforms will provide information on large vessel endothelial dysfunction and injury in specific pathologies.

DEPR:

RT-PCR products from human tissues: placenta, lung, and tongue, were electrophoresed using the CRES/CREA primers specific for EPCR. The procedures were the same as

used for the baboon tissues.

Products corresponding to the membrane isoform of EPCR (mEPCR) and the alternatively-spliced soluble

EPCR isoform (sEPCR) were observed. The products look essentially the same as that seen using the baboon tissues. The only difference is that the placental tissue appears to have additional products.

DEPC:

Detection and Characterization of Soluble EPCR; Physiological Role and Utility as a Marker

DEPC:

Nucleotide and Predicted Protein Structure Analysis of EPCR

DEPC:

Identification of Functional Endothelial Protein C Receptor in Human Plasma

DEPC:

Detection of Soluble EPCR in Urine.

DEPC:

Measurement of Plasma EPCR from Lupus Patients.

DEPC:

Plasma Soluble EPCR in Septic Shock Patients.

DEPC:

Identification of Alternatively Spliced forms of EPCR in Baboon and Human Tissues.

DETL:

TABLE 1 Plasma soluble receptor levels Plasma EPCR Plasma TM
ng/ml ng/ml Normal
volunteers, 133.4 \pm 53.4 35.5 \pm 20.4
n = 20 Systemic lupus 262.1 \pm 154.5* 104.7 \pm 77.5* erythematosus,
(P = 0.0004) (P = 0.0008) n
= 40 Sepsis, n = 24 224.9 \pm 74.5* 39.9 \pm 73.1 (P = 0.00009)
*Significant difference
between the means relative to normal;
unpaired Student's t test.

DETL:

SEQUENCE LISTING (1)
GENERAL INFORMATION: (iii) NUMBER OF SEQUENCES: 8 (2)
INFORMATION FOR SEQ ID NO:1: (i) SEQUENCE
CHARACTERISTICS: (A) LENGTH: 1302 base pairs (B) TYPE: nucleic
acid (C) STRANDEDNESS: single (D)
TOPOLOGY: linear (ii) MOLECULE TYPE: cDNA (iii)
HYPOTHETICAL: NO (iv) ANTI-SENSE: NO (ix) FEATURE:
(A) NAME/KEY: misc.sub.-- feature (B) LOCATION: 1..1302 (D)
OTHER INFORMATION: /note= "Nucleotides
25 through 738 encode the Endothelial Cell Protein Receptor of Sequence
ID No. 2." (xi) SEQUENCE
DESCRIPTION: SEQ ID NO:1:
CAGGTCCGGAGCCTCAACTTCAGGATGTTGACAACATTGCTGCCG
ATACTGCTGCTGTCT60
GGCTGGGCCTTTTGTAGCCAAGACGCCTCAGATGGCCTCCAAAGA
CTTCATATGCTCCAG120
ATCTCCTACTTCCGCGACCCCTATCACGTGTGGTACCAGGGCAAC
CGCTCGCTGGGGGA180
CACCTAACGCACGTGCTGGAAGGCCAGACACCAACACCACGATC
ATTCAGCTGCAGCCC240
TTGCAGGAGCCCGAGAGCTGGGCGCGCAGCAGAGTGGCCTGCA
GTCTACCTGCTCCAG300
TTCCACGGCCTCGTGGCCTGGTGCACCAGGAGCGGACCTTGGCC
TTTCTCTGACCATC360

CGCTGCTTCCTGGGCTGTGAGCTGCCTCCCGAGGGCTCTAGAGCC
CATGTCTTCTTCGAA420

GTGGCTGTGAATGGGAGCTCCTTTGTGAGTTCCGGCCGGAGAGA
GCCTTGTGGCAGGCA480

GACACCCAGGTACCTCCGGAGTGGTCACCTTCACCTGCAGCAG
CTCAATGCCTACAAC540

CGCACTCGGTATGAAGTGGGGAATTCCTGGAGGACACCTGTGTG
CAGTATGTGCAGAAA600

CATATTTCCGCGGAAAAACAGAAAGGGAGCCAAACAAGCCGCTCC
TACACTTCGCTGGTC680

CTGGGCGTCCTGGTGGGCGGTTTCATCATTGCTGGTGTGGCTGTAG
GCATCTTCCTGTGC720

ACAGGTGGACGGCGATGTTAATTACTCTCCAGCCCCGTCAGAAGG
GGCTGGATTGATGGA780

GGCTGGCAAGGGAAAGTTTCAGCTCACTGTGAAGCCAGACTCCCC
AAGTAAACACACAGA840

AGGTTTGGAGTGACAGCTCCTTTCTTCTCCACATCTGCCCACTGA
AGATTTGAGGGAGG900

GGAGATGGAGAGGAGAGGTGGACAAAGTACTTGGTTTGCTAAGA
ACCTAAGAACGTGTAT960

GCTTTGCTGAATTAGTCTGATAAGTGAATGTTATCTATCTTTGTG
GAAAACAGATAATG1020

GAGTTGGGGCAGGAAGCCTATGCGCCATCCTCAAAGACAGACAG
AATCACTGAGGCGT1080

TCAAAAGATATAACCAAATAAACAAGTCATCCACAATCAAATAC
AACATTCAATACTTC1140

CAGGTGTGTCAGACTTGGGATGGGACGCTGATATAATAGGGTAGA
AAGAAGTAACACGAA1200

GAAGTGGTGAAATGTAAAAATCCAAGTCATATGGCAGTGATCAAT
TATTAATCAATTAAT1260

AATATTAATAAAATTTCTTATATTTAAAAA1302
(2) INFORMATION FOR SEQ ID NO:2: (i) SEQUENCE

CHARACTERISTICS: (A) LENGTH: 238 amino acids (B) TYPE: amino
acid (D) TOPOLOGY: linear (ii) MOLECULE

TYPE: protein (iii) HYPOTHETICAL: NO (ix) FEATURE: (A)

NAME/KEY: misc.sub.-- feature (B) LOCATION:

1..365 (D) OTHER INFORMATION: /note= "Endothelial Cell Protein
Receptor encoded by nucleotides 1

through 1302 of Sequence ID No. 1." (ix) FEATURE: (A) NAME/KEY:
Modified-site (B) LOCATION: 1..15

(D) OTHER INFORMATION: /note= "Amino acids 1-15 represent a
putative signal sequence." (ix) FEATURE:

(A) NAME/KEY: Domain (B) LOCATION: 211..236 (D) OTHER
INFORMATION: /note= "Amino acids 211-236

represent a putative transmembrane domain." (ix) FEATURE: (A)

NAME/KEY: Active-site (B) LOCATION:

47..174 (D) OTHER INFORMATION: /note= "Amino acids 47-49, 64-66,
136-138 and 172-174 represent

potential N- glycosylation sites." (ix) FEATURE: (A) NAME/KEY:
Active-site (B) LOCATION: Cys 17 (D)

OTHER INFORMATION: /note=immediately preceeds amino acid
cleavage site (ix) FEATURE: (A) NAME/KEY:

Active-site (B) LOCATION: Gly 201 (D) OTHER INFORMATION:
/note=peptide inserts in alternatively

spliced EPCR (ix) FEATURE: (A) NAME/KEY: Modified-site (B)
LOCATION: 17..186 (D) OTHER INFORMATION:

/note= "Amino acids 17, 114, 118 and 186 represent extracellular cysteine
residues." (xi) SEQUENCE

DESCRIPTION: SEQ ID NO:2:

MetLeuThrThrLeuLeuProLeuLeuLeuSerGlyTrpAlaPhe 151015

CysSerGlnAspAlaSerAspGlyLeuGlnArgLeuHisMetLeuGln 202530

IleSerTyrPheArgAspProTyrHisValTrpTyrGlnGlyAsnAla 354045

SerLeuGlyGlyHisLeuThrHisValLeuGluGlyProAspThrAsn 505560

ThrThrIleLeuGlnLeuGlnProLeuGlnGluProGluSerTrpAla 65707580
ArgThrGlnSerGlyLeuGlnSerTyrLeuLeuGlnPheHisGlyLeu 859095
ValArgLeuValHisGlnGluArgThrLeuAlaPheProLeuThrIle 100105110
ArgCysPheLeuGlyCysGluLeuProProGluGlySerArgAlaHis 115120125
ValPhePheGluValAlaValAsnGlySerSerPheValSerPheArg 130135140
ProGluArgAlaLeuTrpGlnAlaAspThrGlnValThrSerGlyVal 145150155160
ValThrPheThrLeuGlnGlnLeuAsnAlaTyrAsnArgThrArgTyr 165170175
GluLeuArgGluPheLeuGluAspThrCysValGlnTyrValGlnLys 180185190
HisIleSerAlaGluAsnThrLysGlySerGlnThrSerArgSerTyr 195200205
ThrSerLeuValLeuGlyValLeuValGlyGlyPheIleAlaGly 210215220
ValAlaValGlyIlePheLeuCysThrGlyGlyArgArgCys 225230235 (2)

INFORMATION FOR SEQ ID NO:3: (i) SEQUENCE

CHARACTERISTICS: (A) LENGTH: 48 amino acids (B) TYPE: amino
acid (D) TOPOLOGY: linear (ii) MOLECULE

TYPE: protein (iii) HYPOTHETICAL: NO (xi) SEQUENCE

DESCRIPTION: SEQ ID NO:3:

MetMetGlyArgGlyProGlyLysGlnAlaGlyGluArgAlaGlySer 151015

ArgGlnMetAspGlyProGluGlyTrpMetProArgAlaThrArgGly 202530

ProGlnLysGlyValTrpAspArgThrHisAlaAlaSerValSerTrp 354045 (2)

INFORMATION FOR SEQ ID NO:4: (i)

SEQUENCE CHARACTERISTICS: (A) LENGTH: 148 base pairs (B)

TYPE: nucleic acid (C) STRANDEDNESS: single

(D) TOPOLOGY: linear (ii) MOLECULE TYPE: cDNA (iii)

HYPOTHETICAL: NO (iv) ANTI-SENSE: NO (xi)

SEQUENCE DESCRIPTION: SEQ ID NO:4:

ATGATGGGACGGGGCCAGGCTGCAAGCTGGGGAGAGGGCGGGG
TTCCAGACAAATGGAT60

GGACCTGAAGGATGGATGCCTAGAGCAACAAGAGGCCACAGCT
GGGGGTTTGGGACAGA120

ACACACGCAGCTTCAGTCAGTTGGTAA148 (2)

INFORMATION FOR SEQ ID NO:5: (i) SEQUENCE

CHARACTERISTICS: (A) LENGTH: 24 base pairs (B) TYPE:

nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (ii)

MOLECULE TYPE: cDNA (iii)

HYPOTHETICAL: NO (iv) ANTI-SENSE: NO (ix) FEATURE: (A)

NAME/KEY: misc.sub.-- feature (B) LOCATION:

1..24 (D) OTHER INFORMATION: /note= "5'sense primer near end of
exon II." (xi) SEQUENCE DESCRIPTION:

SEQ ID NO:5: TCGTGCCTGGTGCACAGGAGC24 (2)

INFORMATION FOR SEQ ID NO:6: (i) SEQUENCE

CHARACTERISTICS: (A) LENGTH: 24 base pairs (B) TYPE: nucleic
acid (C) STRANDEDNESS: single (D)

TOPOLOGY: linear (ii) MOLECULE TYPE: cDNA (iii)

HYPOTHETICAL: NO (iv) ANTI-SENSE: NO (ix) FEATURE:

(A) NAME/KEY: misc.sub.-- feature (B) LOCATION: 1..24 (D) OTHER

INFORMATION: /note= "3'antisense

primer within exon IV." (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CGCCGTCCACCTGTGCACAGGAAG24 (2)

INFORMATION FOR SEQ ID NO:7: (i) SEQUENCE

CHARACTERISTICS: (A) LENGTH: 22 base pairs (B) TYPE:

nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (ii)

MOLECULE TYPE: cDNA (iii)

HYPOTHETICAL: NO (iv) ANTI-SENSE: NO (ix) FEATURE: (A)

NAME/KEY: misc.sub.-- feature (B) LOCATION:

1..22 (D) OTHER INFORMATION: /note= "5'sense primer near end of
exon III." (xi) SEQUENCE

DESCRIPTION: SEQ ID NO:7: AGCAGCTCAATGCCTACAACCG22

(2) INFORMATION FOR SEQ ID NO:8: (i) SEQUENCE

CHARACTERISTICS: (A) LENGTH: 29 base pairs (B) TYPE: nucleic
acid (C) STRANDEDNESS: single (D)

TOPOLOGY: linear (ii) MOLECULE TYPE: cDNA (iii)

HYPOTHETICAL: NO (iv) ANTI-SENSE: NO (ix) FEATURE:

(A) NAME/KEY: misc.sub.-- feature (B) LOCATION: 1..29 (D) OTHER
INFORMATION: /note= "3'antisense

primer within exon IV." (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CCGTAGAAGGACACGTGTCCACCTGCCGC29

CLPR:

1. An assay for soluble endothelial protein C receptor comprising

CLPR:

3. The assay of claim 1 further comprising the step of correlating the
amount of soluble endothelial
protein C receptor with calibration standards.

CLPR:
8. A kit for detection and measurement of endothelial protein C receptor comprising

OK
N/A
N/A

CLPR:
9. The kit of claim 8 where the antibody has a higher affinity for endothelial protein C receptor including the transmembrane domain than for endothelial protein C receptor not including the transmembrane domain.

CLPR:
10. The kit of claim 8 wherein the antibody is immunoreactive with the insert in an alternatively spliced endothelial protein C receptor.

CLPR:
11. The kit of claim 8 wherein the antibodies block binding of endothelial protein C receptor and activated protein C or protein C.

CLPV:
measuring the amount of soluble endothelial protein C receptor.

CLPV:
an antibody immunoreactive with endothelial protein C receptor,

CLPV:
reagents to detect a reaction between the antibody and endothelial protein C receptor in a sample from a patient, and

CLPV:
standards to correlate the amount of reaction to normal and abnormal levels of endothelial protein C receptor.

ORPL:
Fukudome and Esmon, "Molecular Cloning and Expression of Murine and Bovine Endothelial Cell Protein C/Activated Protein C Receptor (EPCR)—The Structural and Functional Conservation in Human, Bovine and Murine EPCR*," J. Biol. Chem. 270(10):5571-5577 (1995).

9. Document ID: US 5695993 A

L1: Entry 9 of 24

File: USPT

Dec 9, 1997

US-PAT-NO: 5695993
DOCUMENT-IDENTIFIER: US 5695993 A

TITLE: Cloning and regulation of an endothelial cell protein C/activated protein C receptor

DATE-ISSUED: December 9, 1997

INVENTOR-INFORMATION:
NAME

CITY

STATE

ZIP CODE

COUNTRY

Fukudome; Kenji

Oklahoma City

OK

N/A

N/A

Esmon; Charles T.

Oklahoma City

US-CL-CURRENT: 435/325; 435/320.1, 435/69.1, 536/23.5

AB: Human protein C and activated protein C were shown to bind to endothelium specifically, selectively and saturably ($K_d=30$ nM, 7000 sites per cell) in a $Ca^{sup.2+}$ dependent fashion. Expression cloning revealed a 1.3 kb CDNA that coded for a novel type I transmembrane glycoprotein capable of binding protein C. This protein appears to be a member of the CD1/MHC superfamily. Like thrombomodulin, the receptor involved in protein C activation, the endothelial cell protein C receptor (EPCR) function and message are both down regulated by exposure of endothelium to TNF. Identification of EPCR as a member of the CD1/MHC superfamily provides insights into the role of protein C in regulating the inflammatory response, and determination of methods for pharmaceutical use in manipulating the inflammatory response.

L1: Entry 9 of 24

File: USPT

Dec 9, 1997

DOCUMENT-IDENTIFIER: US 5695993 A
TITLE: Cloning and regulation of an endothelial cell protein C/activated protein C receptor

ABPL:
Human protein C and activated protein C were shown to bind to endothelium specifically, selectively and saturably ($K_d=30$ nM, 7000 sites per cell) in a $Ca^{sup.2+}$ dependent fashion. Expression cloning revealed a 1.3 kb CDNA that coded for a novel type I transmembrane glycoprotein capable of binding protein C. This protein appears to be a member of the CD1/MHC superfamily. Like thrombomodulin, the receptor involved in protein C activation, the endothelial cell protein C receptor (EPCR) function and message are both down regulated by exposure of endothelium to TNF. Identification of EPCR as a member of the CD1/MHC superfamily provides insights into the role of protein C in regulating the inflammatory response, and determination of methods for pharmaceutical use in manipulating the inflammatory response.

BSPR:
An endothelial cell protein C binding protein (referred to herein as "EPCR") has been cloned and characterized. The protein is predicted to consist of 238 amino acids, which includes a 15 amino acid signal sequence at the N-terminus, and a 23 amino acid transmembrane region which characterizes the receptor as a type I transmembrane protein. The protein binds with high affinity to both protein C and activated protein C ($K_d=30$ nM) and is calcium dependent. The message and binding function of the receptor are both down regulated by cytokines such as TNF.

BSPR:
These results identify a new member of a complex pathway that, like other members of the pathway, is subject to regulation by inflammatory cytokines, and can therefore be used to modulate inflammatory reactions in which protein C or activated protein C is involved. Inhibition of the inflammatory response can be obtained by infusing soluble EPCR. Alternatively, localizing EPCR to surfaces in

contact with blood will render the surfaces anticoagulant by virtue of the ability of EPCR to bind and concentrate the anticoagulant activated protein C at the surface. Alternatively, the function of EPCR can be enhanced by overexpressing the EPCR in endothelium that could be used to coat vascular grafts in patients with vascular disease or on stents in cardiac patients.

DRPR:

FIGS. 3A and 3B are flow cytometric analyses of F1-APC binding to 293T cells transfected with a cDNA clone of EPCR. Cells were transfected with a clone EPCR/pEF-BOS or pEF-BOS (negative control) by the calcium/phosphate method. After 24 h, cells were harvested and F1-APC binding was performed in the absence (dotted lines) or presence of 1.3 mM CaCl₂ (solid lines).

DRPR:

FIG. 4 is the predicted protein structure of EPCR based on nucleotide sequence Sequence ID No. 1, predicted amino acid sequence, Sequence ID No. 2, and a hydropathy plot of EPCR. The signal sequence and transmembrane region are indicated with the solid bars.

DRPR:

FIG. 5 is a comparison of the amino acid sequence of EPCR to the amino acid sequences of other members of the CD1 family and CCD41. The EPCR sequence, Sequence ID No. 2, is shown in the first line and compared to murine CCD41, Sequence ID No. 3, (second line), human CD1d, Sequence ID No. 4, (third line) and murine CD1.2, Sequence ID No. 5, (fourth line). Identities with EPCR are indicated by open boxes. Residues that are conserved between EPCR and all of the human CD1 family members are indicated by a double asterisk. Residues shared with one or more members of the CD1 family are indicated by a single asterisk.

DRPR:

FIG. 6 is a comparison of the amino acid sequence of human EPCR, Sequence ID No. 2, (first line) to the amino acid sequence of murine EPCR, Sequence ID No. 6, (second line). Identities are indicated by boxes. Similarities are indicated with an asterisk.

DEPR:

Human protein C and activated protein C are shown to bind to endothelium specifically, selectively and saturably ($K_d=30$ nM, 7000 sites per cell) in a Ca²⁺ dependent fashion. FL-APC binding to various human cell lines were examined, and found that the binding was HUVEC specific. A human kidney cell line transformed with SV40 large T antigen, 293T cells, expressed very few of these binding sites. A HUVEC cDNA library was constructed using the powerful mammalian expression vector, pEF-BOS (Mizushima and Nagata, (1990) *Nucleic Acids Res.* 18, 5322). Plasmid DNA was prepared from subpools of independent colonies (2,500 colonies per pool), and transfected into 293T cells, using the method of Kaisho et al., (1994) *Proc. Natl. Acad. Sci. (USA)* 91, 5325. FL-APC binding was analyzed on a flow cytometer. One of eight subpools gave a positive signal. This subpool was divided into 20 subpools and rescreened. After three rounds of enrichment, one positive clone, EPCR-1, was isolated. EPCR-1 carries a 1.3 kb insert. When transfected into 293T cells, this clone was capable of expressing the calcium-dependent binding site for FL-APC on the 293T cell surface.

DEPR:

Expression cloning revealed a 1.3 kb cDNA that coded for a type I transmembrane glycoprotein capable of binding protein C. This protein appears to be a member of the CD1/MHC superfamily. Like thrombomodulin, the receptor involved in protein C activation, the

endothelial cell protein C

receptor (EPCR) function and message are both down regulated by exposure of endothelium to TNF.

Identification of EPCR as a member of the CD1/MHC superfamily provides insights into the role of this receptor for protein C in regulating the inflammatory response.

DEPR:

Total RNAs (15 μ g) from various cells were isolated, electrophoresed through formaldehyde agarose gels and transferred to a nylon membrane (Hybond-N.TM., Amersham). The 483 bp Xba I fragment from the 5' end of the EPCR cDNA was labeled by random priming according to the manufacturer's instructions (Multiprime.TM. DNA labeling system, Amersham) and used for hybridization.

DEPR:

Nucleotide and Predicted Protein Structure Analysis of EPCR

DEPR:

The cDNA is predicted to code for a protein of 238 amino acids (Sequence ID No. 2), which includes a 15 amino acid signal sequence (yon Heijne, (1986) *Nucleic Acids Res.* 14, 4683-4690) at the N-terminal. Therefore, the mature protein is predicted to contain 223 amino acids. Sequence ID No. 2 is the predicted amino acid sequence of EPCR. Amino acids 1-15 of Sequence ID No. 2 (MLTTLPIILLSGWA) are the putative signal sequence determined by the method of yon Heijne (yon Heijne, 1986). Amino acids 211-236 of Sequence ID No. 2 (LVGLVVGGFILAGVAVGIFLCTGGR) are the putative transmembrane domain. Potential N-glycosylation sites are present at amino acids 47-49, 64-66, 136-138, and 172-174 of Sequence ID No. 2. Extracellular cysteine residues are present at amino acids 17, 114, 118, and 186 of Sequence ID No. 2. A potential transmembrane region (Engelman et al., (1986) *Annu. Rev. Biophys. Biophys. Chem.* 15, 321-53) consisting of 23 amino acids was identified at the C-terminal end (beginning at amino acid 216 of Sequence ID No. 2).

DEPR:

DNA and protein database searches revealed that the sequence is related to the centrosome-associated, cell cycle dependent murine protein, CCD41, also referred as centrocyclin (Rothbarth et al., (1993) *J. Cell Sci.* 104, 19-30), as shown by FIG. 5. The similarity in the published sequence of murine CCD41 with human EPCR led to the cloning and sequencing of the murine EPCR. The sequence of murine EPCR is shown in FIG. 6. It is distinct from the published sequence of CCD41.

DEPR:

The EPCR amino acid sequence was also related to, but quite distinct from, the CD1/MHC superfamily and the murine CD1.2, as also shown by FIG. 5. Based on the homology to CD1/MHC, it is likely that EPCR contains two domains consisting of residues 17-114 and 118-188. Of the CD1 family members, CD1d is the most similar to EPCR. In the mouse, CCD41 is associated exclusively with the centrosome during G₁ but becomes detectable elsewhere during the cell cycle, reaching a maximum during G₂, except during the G₂/M phase (Rothbarth et al., 1993). EPCR expression appears restricted to endothelium, which would not be expected for a cell cycle associated protein.

DEPR:

The identification of the protein C receptor on endothelium suggests that the endothelial cell binds protein C/APC through three distinct mechanisms. In addition to EPCR, protein S can bind APC/protein

C on negatively charged membrane surfaces that include the endothelium (Stern et al., (1986) J.

Biol. Chem. 261, 713-718), but this is not cell type specific (Dahlback et al., 1992).

Thrombomodulin in complex with thrombin can bind protein C and APC (Hogg et al., 1992). On

endothelium, the protein S binding sites (Nawroth and Stern, (1986) J.

Exp. Med. 163, 740-745),

thrombomodulin (Esmon, 1989) and EPCR are all down regulated by cytokines, indicating that

inflammation can impair protein C pathway function at multiple levels.

DEPR:

The homology to the CD1/MHC family of proteins is especially interesting since it provides

indications as to the function of EPCR. The CD1/MHC family has three extracellular domains termed

.alpha.1,2 and 3. The extracellular domain of EPCR contains four Cys residues that appear to

correspond to two distinct domains. EPCR lacks the third domain of the CD1/MHC family, but the two

domains have significant homology to the .alpha.1 and .alpha.2 domains of the CD1 protein family and

the .alpha.2 domain of the MHC class I protein, suggesting that these proteins evolved from a common

ancestor. The first domain of EPCR, residues 17-114, contains two potential N glycosylation sites

and is rich in .beta. strand structure, suggesting that it may form a .beta. sheet. Despite the

.beta. strand structure, consensus sequences (Williams and Barclay, (1988) Ann. Rev. Immunol. 6,

381-405) for the immunoglobulin superfamily of receptors are absent. The second domain of EPCR,

residues 118-188, contains two additional N glycosylation sites and, like the CD1/MHC family, this

domain is predicted to have limited .beta. structure.

DEPR:

In vitro studies have suggested anti-inflammatory activities for APC. For instance, an unusual

carbohydrate sequence on protein C can inhibit inflammatory cell adhesion to selectins (Grinnell et

al., (1994) Glycobiology, 4, 221-226) Modest inhibitory effects of APC have been reported on TNF

production (Grey et al., (1993) Transplant. Proc. 25, 2913-2914). EPCR could contribute to these

anti-inflammatory mechanisms. Since the homologous protein family, CD1, can be linked to CD8

(Ledbetter et al., (1985) J. Immunol. 134, 4250-4254), it is also possible that the proteins C

receptor is linked to another protein and signal through this second protein. One of the CD1 family

members, CD1d, has been reported to promote T cell responses, possibly involving binding to CD8

(Panja et al., (1993) J. Exp. Med. 178, 1115-1119). CD1b has recently been reported to serve as an

antigen presenting molecule (Porcelli et al., (1992) Nature 360, 593-597). The ability to bind

protein C/APC could then be linked either directly or indirectly to signalling via direct

interaction with cells of the immune system. Since the MHC class of proteins is involved in

presentation of proteins to cell receptors, the concept of presentation of protein C/APC to

inflammatory cells as a means of elaborating anti-inflammatory activity may also be involved. This

includes modulation of enzyme specificity such as occurs with thrombin-thrombomodulin interaction

(Esmon, 1989). In this case, the EPCR-APC complex might cleave biologically active peptides from

unknown substrates.

DEPR:

EPCR mRNA Levels and APC Binding

DEPR:

To determine the cellular specificity of EPCR expression, the intensity of FL-APC binding to HUVEC

was compared to several human cell lines. F1-APC bound strongly only to HUVEC, and not to any of the

T, B, or monocytic cell lines tested. Cells were incubated at room temperature without or with 160

nM F1-APC in the presence of 1.3 mM CaCl₂. Binding was analyzed by flow cytometry. Slight

binding was demonstrated with the osteosarcoma line, HOS and the epidermoid carcinoma cell line,

HEp-2.

DEPR:

Total RNA was extracted from these cells and hybridized with the EPCR cDNA probe for Northern Blot

Analysis. EPCR mRNA was detected by Northern blot analysis for HUVEC, Jurkat, HEp-2, Raji, HOS, and

U937. Among the cells lines tested, EPCR mRNA was detected at high levels only in HUVEC. The

calculated mRNA size of 1.3 kb was identical to the size of the isolated cDNA. After prolonged

exposure, a weak signal was also detected with the osteosarcoma cell line HOS and monocyte cell line

U937. Thus, both APC binding and EPCR mRNA expression are very specific for endothelium.

DEPR:

Effects of TNF on APC Binding and EPCR mRNA Levels

DEPR:

Several other members of the protein C anticoagulant pathway are subject to regulation by

inflammatory cytokines (Esmon, 1989). For instance, endothelial cell surface thrombomodulin

expression and message are known to be reduced by exposure of the cells to TNF (Conway and

Rosenberg, 1988; Lentz et al., 1991). To determine if a similar process occurs with EPCR, HUVEC were

treated with TNF and APC binding and expression of EPCR mRNA were examined. APC binding to HUVEC

decreased in a time dependent fashion. EPCR activity decreased more rapidly than thrombomodulin

antigen. HUVEC were cultured for 0, 6, 24 and 48 hr, in the presence of TNF-.alpha. (10 ng/ml).

Cells were harvested and residual F1-APC binding or thrombomodulin (TM) expression was analyzed by

flow cytometry. Cell surface TM was stained with an anti-TM murine monoclonal antibody and

FITC-conjugated anti-mouse IgG. The negative control is without added fluorescent ligand.

DEPR:

HUVEC were treated with 10 ng/ml of TNF-.alpha. for 0, 0.5, 1, 2, 3, 6, 10 and 24 hr, and message

was extracted and detected as described above. The results demonstrated that the concentration of

EPCR mRNA was also reduced by TNF treatment. Message levels and APC binding activity decreased in

parallel. Therefore, the TNF mediated down-regulation of APC binding to endothelium probably occurs

at the level of mRNA expression.

DEPR:

Enhancement of inflammatory responses by blocking binding of endogenous molecules to EPCR can be

achieved by administration of compounds binding to the receptor to a subject in need of inhibition.

The degree of binding is routinely determined using assays such as those described above. Compounds

which are effective include antibodies to the protein, fragments of antibodies retaining the binding

regions, and peptide fragments of APC which include the Gla region. Inhibition of the inflammatory

response could be obtained by infusing soluble EPCR. Alternatively, localizing EPCR to surfaces in

contact with blood would render the surfaces anticoagulant by virtue of the ability of EPCR to bind

and concentrate the anticoagulant APC at the surface. Alternatively, the function of EPCR could be

enhanced by overexpressing the EPCR in endothelium used to coat

vascular grafts in patients with
vascular disease or on stents in cardiac patients.

DEPR:

Patients with thrombosis or hyperinflammatory conditions could be screened for defects in the EPCR gene. Sequence ID No. 1, and consecutive portions thereof of at least about seven nucleotides, more preferably fourteen to seventeen nucleotides, most preferably about twenty nucleotides, are useful in this screening using hybridization assays of patient samples, including blood and tissues. Screening can also be accomplished using antibodies, typically labelled with a fluorescent, radiolabelled, or enzymatic label, or by isolation of target cells and screening for binding activity, as described in the examples above. Typically, one would screen for expression on either a qualitative or quantitative basis, and for expression of functional receptor. Labelling can be with .sup.32 P, .sup.35 S, fluorescein, biotin, or other labels routinely used with methods known to those skilled in the art for labelling of proteins and/or nucleic acid sequences.

DEPR:

In cases where inflammatory mediators or vascular disease down regulate EPCR, it would be advantageous to increase its concentration in vivo on endothelium. The binding assays described here and the gene sequence allow assays for increased EPCR expression. Similar approaches have been taken with thrombomodulin and investigators have shown that cyclic AMP (Maruyama, I. et al. (1991) Thrombosis Research 61, 301-310) and interleukin 4 (Kapiotis, S. et al., (1991) Blood 78, 410-415) can elevate thrombomodulin expression. The ability to screen such drugs or drugs that block TNF down regulation of EPCR provide an approach to elevating EPCR expression in vivo and thus enhancing anticoagulant and anti-inflammatory activity.

DEPR:

Studies based on inhibition of binding are predictive for indirect effects of alteration of receptor binding. For example, inhibition of binding of APC or increased expression of TNF is predictive of inhibition of EPCR function.

DEPR:

As described herein, a variety of compounds can be used to inhibit or enhance expression of the EPCR. The nature of the disorder will determine if the expression should be enhanced or inhibited. For example, based on the studies involving the use of an anti-protein C antibody in combination with cytokine, it should be possible to treat solid tumors by enhancing an inflammatory response involving blocking of protein C or activated protein C binding to an endothelial cell protein C/activated protein C receptor by administering to a patient in need of treatment thereof an amount of a compound blocking binding of protein C or activated protein C to the receptor. Similarly, it should be possible to treat disorders such as gram negative sepsis, stroke, thrombosis, septic shock, adult respiratory distress syndrome, and pulmonary emboli using a method for inhibiting an inflammatory response involving administration of EPCR or EPCR fragments or substances that upregulate EPCR expression to a patient in need of treatment thereof.

DEPL:

I. Cloning and Characterization of EPCR

DEPL:

II. Modulation of Inflammation using EPCR.

ORPL:

Fukudome, K. et al., "Molecular Cloning and Expression of Murine and Bovine Endothelial Cell Protein C/activated Protein C Receptor (EPCR)", J. Biological Chemistry, vol. 270, no. 10, pp. 5571-5577 (1995).

10. Document ID: US 5548796 A

L1: Entry 10 of 24

File: USPT

Aug 20, 1996

US-PAT-NO: 5548796

DOCUMENT-IDENTIFIER: US 5548796 A

TITLE: Method of automatic retransmission of frames in a local area network

DATE-ISSUED: August 20, 1996

INVENTOR-INFORMATION:
NAME

CITY	STATE	ZIP CODE	COUNTRY
Ketchum; Kevin D.	Folsom	CA	N/A
		N/A	N/A

US-CL-CURRENT: 710/52; 370/447

AB: A configurable network interface controller that provides for the automatic retransmission of collided Ethernet frames from a local RAM while observing two modes of operation: (1) retransmission of as much of the frame as possible without violating latency requirements and (2) first guaranteeing the safe retransmission of the first 64 bytes and then returning to observation of the latency requirements.

L1: Entry 10 of 24

File: USPT

Aug 20, 1996

DOCUMENT-IDENTIFIER: US 5548796 A

TITLE: Method of automatic retransmission of frames in a local area network

DRPR:

FIG. 38 is a table that describes the EEPROM Configuration Register (EPCR) of the SCENIC device.

DEPC:

EEPROM Configuration Register (EPCR)

11. Document ID: US 5513376 A

L1: Entry 11 of 24

File: USPT

Apr 30, 1996

US-PAT-NO: 5513376

DOCUMENT-IDENTIFIER: US 5513376 A

TITLE: Method of operating an extension FIFO in another device when it is full by periodically re-initiating a write operation until data can be transferred

DATE-ISSUED: April 30, 1996

INVENTOR-INFORMATION:
NAME

CITY

STATE

ZIP CODE

COUNTRY

Lohmeyer; Michael G.

San Jose

CA

N/A

N/A

US-CL-CURRENT: 710/53; 365/220, 365/221, 710/2, 710/34, 710/52

AB: A configurable network interface controller provides a multi-chip FIFO extension protocol. Utilizing this protocol, FIFOs that are physically separated (e.g., in separate chips) can be made to operate as though they are a single FIFO.

L1: Entry 11 of 24

File: USPT

Apr 30, 1996

DOCUMENT-IDENTIFIER: US 5513376 A

TITLE: Method of operating an extension FIFO in another device when it is full by periodically re-initiating a write operation until data can be transferred

DRPR:

FIG. 38 is a table that describes the EEPROM Configuration Register (EPCR) of the SCENIC device.

DEPC:

EEPROM Configuration Register (EPCR) (A<7:0>-0Ah)

12. Document ID: US 5495593 A

L1: Entry 12 of 24

File: USPT

Feb 27, 1996

US-PAT-NO: 5495593

DOCUMENT-IDENTIFIER: US 5495593 A

TITLE: Microcontroller device having remotely programmable EPROM and method for programming

DATE-ISSUED: February 27, 1996

INVENTOR-INFORMATION:
NAME

CITY

STATE

ZIP CODE

COUNTRY

Elmer; Thomas I.

Santa Clara

CA

N/A

N/A

Nguyen; Tuan T.

Milpitas

CA

N/A

N/A

Lin; Rung-Pan

San Jose

CA

N/A

N/A

US-CL-CURRENT: 711/103; 711/147

AB: A microcontroller device on a single integrated circuit including a central processing unit, an associated data bus and an electrically-programmable nonvolatile memory is disclosed. The nonvolatile memory contains the applications program and may be remotely programmed by way of a communication port, such as a universal asynchronous, receiver/transmitter (UART) device, utilizing a separate host computer. A second nonvolatile memory is provided which contains a control program which is executed by the central processing unit for carrying out the programming of the electrically-programmable nonvolatile memory utilizing data and address information received from the host computer over the communications port.

L1: Entry 12 of 24

File: USPT

Feb 27, 1996

DOCUMENT-IDENTIFIER: US 5495593 A

TITLE: Microcontroller device having remotely programmable EPROM and method for programming

DEPR:

The subject microcontroller device includes a main bus 72 used to transport addresses and data, which is coupled to, among other devices, core 14, UART 22, UART programming control ROM 20, RAM 13, timer/counter 80, EPROM Data Register (EPDR) 74, Memory Address Register (MAR) 68, EPROM Address Register (EPAR) 66, EPROM Control Register (EPCR) 64, and the data out port of EPROM 18. The data in port of EPROM 18 is connected to the output of EPDR 74 via EPROM data in bus 76.

DEPR:

The EPROM program control register (EPCR) 64 is used to control the programming of EPROM 18. The outputs of register EPCR 64 include signal NPGM on line 82, which is a programming strobe, connected to the NPGM input of EPROM 18. As will be explained later in greater detail, an NPGM strobe is produced when an address of EPROM 18 is to be programmed with data. The outputs of register EPCR 64 also include signal ME on line 81 which controls address multiplexer 70, as explained previously.

DEPR:

UART Programming Control ROM 20 contains 256 bytes and functions as

a shadow memory, which means the memory cannot be accessed during the normal run mode, but only in the UART program mode and the run time programming mode. In the UART programming mode, the 256 bytes of UART Programming Control ROM 20 are assigned addresses 0300 (hex) through 03FF (hex) of the processing unit 15 address space.

Registers EPDR 74, EPAR 66, and EPCR 64 are also mapped in the shadow memory, meaning they cannot be accessed in normal run mode, but only in UART and run time programming modes. EPDR 74 is mapped at address 00FA (hex) in shadow memory. EPAR 66 is mapped at address 00FC (hex) in shadow memory. EPCR 64 is mapped at address 00FE (hex) in shadow memory. Thus, the "shadow memory" addresses are part of the processor address space in the UART programming mode and in the run time programming mode but are removed from the address space in the normal run mode.

DEPR:

The second type of command is the Prescaler Information Command which controls the duration of the internal program pulse NPGM on line 82 produced by register EPCR 64 (FIG. 4). The Prescaler

Information Command is a single byte command which includes four bits which identify the command

type and four bits which select a prescaler value for microcontroller 12. The prescaler value can vary from decimal 16 (2.sup.4) to 131,072 (2.sup.17) and is selected according to the prescaler

command as indicated by Table 1, below.

DEPR:

As previously noted, EPROM 18 is preferably programmed utilizing an interactive programming sequence. Details of the interactive programming sequence are shown in the FIG. 5 flow chart.

Initially, a software counter is set to a value of 20, as represented by blocks 90 and 92. Next, a first attempt to program EPROM 18 with one byte of received data (block 94) is made by generating one program pulse NPGM (FIG. 4). The EPROM Control Register (EPCR) 64 (FIG. 4) will be caused to produce the program pulse NPGM on line 82 by software executed from control ROM 20. The duration of this pulse will be determined by the prescaler value P obtained from a previously received Prescaler

Information Command (Table 1). The interaction of software executing from control ROM 20 with timer/counter 80 (FIG. 4) will determine the duration of the NPGM pulse in accordance with the value P and equation (1). The data to be programmed are held in EPDR 74. (FIG. 4).

DEPR:

Run time programming is accomplished by first increasing voltage Vpp on element 32 (FIG. 2) to +12.75 volts. Accordingly, a system design must be implemented to automatically increase Vpp in the event run time programming is to be carried out. Voltage sensor 34 causes the UART Program Mode (UPM) signal on line 62 to be produced thereby enabling the data register EPCR 74 (FIG. 4), control register EPDR 64, control ROM 20 and address register EPAR 66. Core 14 may then cause certain sections of software stored in control ROM 20 to program predetermined critical data to predetermined EPROM 18 addresses for later evaluation.

L1: Entry 13 of 24

File: USPT

Jan 21, 1986

US-PAT-NO: 4566124

DOCUMENT-IDENTIFIER: US 4566124 A

TITLE: Pattern reading system

DATE-ISSUED: January 21, 1986

INVENTOR-INFORMATION:

NAME

CITY

STATE

ZIP CODE

COUNTRY

Yamamoto; Kazuhiko

Ushiku

N/A

N/A

JPX

Saito; Taiichi

Ibaraki

N/A

N/A

JPX

US-CL-CURRENT: 382/185; 382/197, 382/316

AB: A pattern reading system by line segment approximation comprising the steps of tracing the contour and simultaneously, seeking out as candidate extreme points the points at which the inner products of coordinate point vectors and directional vectors at coordinate points of the contour being traced are largest, and feeding out these candidate extreme points as real extreme points when the differences between the inner products of the direction vectors and the inner products of the candidate extreme points are greater than an allowance set in advance.

L1: Entry 13 of 24

File: USPT

Jan 21, 1986

DOCUMENT-IDENTIFIER: US 4566124 A

TITLE: Pattern reading system

DEPR:

Preparatory to starting the tracing, in the output switches (Sw.) 10 for extreme point with respect to 16 directions, those for the directions D.sub.5 .about.D.sub.12 are turned ON and the other switches are turned OFF. An inner product calculator (IPC) 11 is operated to calculate projections (a.sub.h, e.sub.i) of the start point for 16 directions. The found values of (a.sub.h, e.sub.i) for the 16 directions are transferred to relevant extreme point candidate inner product registers 12 and start point coordinates for all the directions are set in extreme point candidate coordinate registers (EPCR) 13.

DEPR:

When the aforementioned sum exceeds the allowance, a comparator (CM) 18 issues a signal which causes the x- and y-coordinates of the extreme point candidate coordinate register (EPCR) 13 to be fed out into the extreme point output register (EPOR) 19 and also causes the extreme point output switch 10 to be turned ON in the D.sub.i -direction and OFF in the D.sub.r -direction.

When the sum is less than the allowance, the inner product (a.sub.h, e.sub.i) of the extreme candidate inner product register 12 and the inner product (a.sub.j, e.sub.i) of the inner product register 16 are compared with each other by a comparator (CM) 20. The contents in the D.sub.i -direction of the extreme point candidate inner product register 12 are exchanged for those of the current point a.sub.j of the contour when the inner product (a.sub.h, e.sub.i) is smaller than the inner product (a.sub.j, e.sub.i). Nothing takes place when the inner product (a.sub.h, e.sub.i) is greater than or equal to the inner product (a.sub.j, e.sub.i). With respect to the D.sub.i -direction in which the extreme point output switch 10 is ON, the inner product (a.sub.j, e.sub.i) and the x- and y-coordinates of the current point of the contour are forcedly set respectively in the registers 12 and 13.

14. Document ID: US 5852171 A

L1: Entry 14 of 24

File: EPAB

Dec 22, 1998

PUB-NO: US00585217A
DOCUMENT-IDENTIFIER: US 5852171 A
TITLE: Cloning and regulation of an endothelial cell protein C/activated protein C receptor

PUBN-DATE: December 22, 1998

INVENTOR-INFORMATION:
NAME

FUKUDOME, KENJI

COUNTRY

US

ESMON, CHARLES T

US

INT-CL (IPC): C07K 14/705
EUR-CL (EPC): C07K014/705

AB: Human protein C and activated protein C were shown to bind to endothelium specifically, selectively and saturably (Kd=30 nM, 7000 sites per cell) in a Ca2+ dependent fashion. Expression cloning revealed a 1.3 kb CDNA that coded for a novel type I transmembrane glycoprotein capable of binding protein C. This protein appears to be a member of the CD1/MHC superfamily. Like thrombomodulin, the receptor involved in protein C activation, the endothelial cell protein C receptor (EPCR) function and message are both down regulated by exposure of endothelium to TNF. Identification of EPCR as a member of the CD1/MHC superfamily provides insights into the role of protein C in regulating the inflammatory response, and determination of methods for pharmaceutical use in manipulating the inflammatory response.

L1: Entry 14 of 24

File: EPAB

Dec 22, 1998

DOCUMENT-IDENTIFIER: US 5852171 A
TITLE: Cloning and regulation of an endothelial cell protein C/activated protein C receptor

FPAR:

Human protein C and activated protein C were shown to bind to endothelium specifically, selectively and saturably (Kd=30 nM, 7000 sites per cell) in a Ca2+ dependent fashion. Expression cloning revealed a 1.3 kb CDNA that coded for a novel type I transmembrane glycoprotein capable of binding protein C. This protein appears to be a member of the CD1/MHC superfamily. Like thrombomodulin, the receptor involved in protein C activation, the endothelial cell protein C receptor (EPCR) function and message are both down regulated by exposure of endothelium to TNF. Identification of EPCR as a member of the CD1/MHC superfamily provides insights into the role of protein C in regulating the inflammatory response, and determination of methods for pharmaceutical use in manipulating the inflammatory response.

15. Document ID: US 5804392 A

L1: Entry 15 of 24

File: EPAB

Sep 8, 1998

PUB-NO: US005804392A
DOCUMENT-IDENTIFIER: US 5804392 A
TITLE: Diagnostic assays using soluble endothelial cell protein C/activated protein C receptor

PUBN-DATE: September 8, 1998

INVENTOR-INFORMATION:
NAME

ESMON, CHARLES T

COUNTRY

US

STEARNS-KUROSAWA, DEBORAH J

US

KUROSAWA, SHINICHIRO

US

INT-CL (IPC): G01N 33/53; G01N 33/564; C07K 16/28

AB: CHG DATE=19990617 STATUS=O>Plasma EPCR has been isolated, characterized and shown to block cellular protein C activation and APC anticoagulant activity. Plasma EPCR appears to be about 43,000 daltons and circulates at approximately 100 ng/ml (98.4+/-27.8 ng/ml, n=22). Plasma EPCR bound activated protein C with an affinity similar to that of recombinant soluble EPCR (Kdapp approximately 30 nM), and inhibits both protein C activation on an endothelial cell line and APC anticoagulant activity in a one-stage factor Xa clotting assay. Soluble plasma EPCR appears to attenuate the membrane-bound EPCR augmentation of protein C activation and the anticoagulant function of activated protein C. Soluble EPCR has also been detected in urine. Levels of soluble EPCR can rise in inflammatory disease associated with vascular injury and appear to be correlated with inflammation and disease states associated with abnormal

coagulation. Since EPCR expression is restricted to larger vessels and is usually negative in capillaries, these observations provide a mechanism for analyzing injury/stimulation of large vessel endothelial cells.

L1: Entry 15 of 24

File: EPAB

Sep 8, 1998

DOCUMENT-IDENTIFIER: US 5804392 A
TITLE: Diagnostic assays using soluble endothelial cell protein C/activated protein C receptor

FPAR:
CHG DATE=19990617 STATUS=O>Plasma EPCR has been isolated, characterized and shown to block cellular protein C activation and APC anticoagulant activity. Plasma EPCR appears to be about 43,000 daltons and circulates at approximately 100 ng/ml (98.4+/-27.8 ng/ml, n=22). Plasma EPCR bound activated protein C with an affinity similar to that of recombinant soluble EPCR (Kdapp approximately 30 nM), and inhibits both protein C activation on an endothelial cell line and APC anticoagulant activity in a one-stage factor Xa clotting assay. Soluble plasma EPCR appears to attenuate the membrane-bound EPCR augmentation of protein C activation and the anticoagulant function of activated protein C. Soluble EPCR has also been detected in urine. Levels of soluble EPCR can rise in inflammatory disease associated with vascular injury and appear to be correlated with inflammation and disease states associated with abnormal coagulation. Since EPCR expression is restricted to larger vessels and is usually negative in capillaries, these observations provide a mechanism for analyzing injury/stimulation of large vessel endothelial cells.

16. Document ID: WO 9820041 A1

L1: Entry 16 of 24

File: EPAB

May 14, 1998

PUB-NO: WO009820041A1
DOCUMENT-IDENTIFIER: WO 9820041 A1
TITLE: ENDOTHELIUM SPECIFIC EXPRESSION REGULATED BY EPCR CONTROL ELEMENTS

PUBN-DATE: May 14, 1998

INVENTOR-INFORMATION:
NAME

ESMON, CHARLES T

GU, JIAN-MING

COUNTRY

N/A

N/A

INT-CL (IPC): C07K 14/705
EUR-CL (EPC): C07K014/705

AB: CHG DATE=19990617 STATUS=O>The promoter of the EPCR gene has been isolated from both murine (SEQ. ID No. 1) and human (SEQ. ID No. 2) genomic libraries.

The promoter has been demonstrated to include a region which results in selective expression in endothelial cells, between -1 and -220 based on the positions relative to the ATG encoding the first amino acid of the murine EPCR protein (nucleotides 3130 to 3350 of SEQ. ID No. 1), and a region which selectively results in expression in large vessel endothelial cells, as opposed to all endothelial cells, located between -700 and -1080 (nucleotides 2270 to 2840 of SEQ. ID No. 1).

A thrombin responsive element has been identified in the EPCR promoter, from -337 to -345 in the murine promoter (nucleotides 3007 to 3014 SEQ. ID No. 1) and from -360 to -368 (nucleotides 2722 to 2729 SEQ. ID No. 2) in the human promoter. The sequence is CCCACCCC (SEQ. ID No. 3). A serum response element has also been identified between -280 and -350 (nucleotides 2990 to 3061 of SEQ. ID No. 1). The regulatory sequences present in the EPCR promoter can be used in combination with genes encoding other proteins, as well as shorter oligonucleotides, to increase expression by exposure to thrombin or serum or to effect selective expression in endothelial cells generally or preferentially in endothelial cells of the large blood vessels.

The gene control elements include elements responsive to environmental stimuli (either thrombin or serum); and information to determine distribution of the desired protein expression (large vessels). Therapeutic strategies include the use of the minimal promoter (-220 to -1) for expression in all endothelial cells, for example, for any kind of gene therapy where systemic distribution is desired; the use of a promoter including an environmental stimuli response element(s), for use in delivery of agents whose expression should be increased during times of increased thrombin/platelet activation or during regional trauma; the use of the minimal promoter including an environmental stimuli response element and the element directing expression to large vessel endothelium, where a response to regional trauma is desirable but only in large vessel endothelium, and the use of the minimal promoter and element directing expression to large vessel endothelium, where expression is specifically targeted to large vessel endothelium but is not increased in response to any particular stimuli.

L1: Entry 16 of 24

File: EPAB

May 14, 1998

DOCUMENT-IDENTIFIER: WO 9820041 A1
TITLE: ENDOTHELIUM SPECIFIC EXPRESSION REGULATED BY EPCR CONTROL ELEMENTS

FPAR:

CHG DATE=19990617 STATUS=O>The promoter of the EPCR gene has been isolated from both murine (SEQ. ID No. 1) and human (SEQ. ID No. 2) genomic libraries. The promoter has been demonstrated to include a region which results in selective expression in endothelial cells, between -1 and -220 based on the positions relative to the ATG encoding the first amino acid of the murine EPCR protein (nucleotides 3130 to 3350 of SEQ. ID No. 1), and a region which selectively results in expression in large vessel endothelial cells, as opposed to all endothelial cells, located between -700 and -1080 (nucleotides 2270 to 2840 of SEQ. ID No. 1). A thrombin responsive element has been identified in the EPCR

promoter, from -337 to -345 in the murine promoter (nucleotides 3007 to 3014 SEQ. ID No. 1) and from -360 to -368 (nucleotides 2722 to 2729 SEQ. ID No. 2) in the human promoter. The sequence is

CCCACCCC (SEQ. ID No. 3). A serum response element has also been identified between -280 and -350 (nucleotides 2990 to 3061 of SEQ. ID No. 1). The regulatory sequences present in the EPCR promoter

can be used in combination with genes encoding other proteins, as well as shorter oligonucleotides,

to increase expression by exposure to thrombin or serum or to effect selective expression in

endothelial cells generally or preferentially in endothelial cells of the large blood vessels. The

gene control elements include elements responsive to environmental stimuli (either thrombin or

serum); and information to determine distribution of the desired protein expression (large vessels).

Therapeutic strategies include the use of the minimal promoter (-220 to -1) for expression in all

endothelial cells, for example, for any kind of gene therapy where systemic distribution is desired;

the use of a promoter including an environmental stimuli response element(s), for use in delivery of

agents whose expression should be increased during times of increased thrombin/platelet activation

or during regional trauma; the use of the minimal promoter including an environmental stimuli

response element and the element directing expression to large vessel endothelium, where a response

to regional trauma is desirable but only in large vessel endothelium, and the use of the minimal

promoter and element directing expression to large vessel endothelium, where expression is

specifically targeted to large vessel endothelium but is not increased in response to any particular

stimuli.

appears to be a member

of the CD1/MHC superfamily. Like thrombomodulin, the receptor involved in protein C activation,

the endothelial cell protein C receptor (EPCR) function and message are both down regulated by

exposure of endothelium to TNF. Identification of EPCR as a member of the CD1/MHC superfamily

provides insights into the role of protein C in regulating the inflammatory response, and

determination of methods for pharmaceutical use in manipulating the inflammatory response.

L1: Entry 17 of 24

File: EPAB

Dec 9, 1997

DOCUMENT-IDENTIFIER: US 5695993 A

TITLE: Cloning and regulation of an endothelial cell protein C/activated protein C receptor

FPAR:

CHG DATE=19990617 STATUS=O>Human protein C and activated protein C were shown to bind to endothelium specifically, selectively and saturably (Kd=30 nM, 7000 sites per cell) in a Ca2+ dependent fashion.

Expression cloning revealed a 1.3 kb CDNA that coded for a novel type I transmembrane glycoprotein capable of binding protein C. This protein appears to be a member of the CD1/MHC superfamily. Like

thrombomodulin, the receptor involved in protein C activation, the endothelial cell protein C

receptor (EPCR) function and message are both down regulated by exposure of endothelium to TNF.

Identification of EPCR as a member of the CD1/MHC superfamily provides insights into the role of protein C in regulating the inflammatory response, and determination of methods for pharmaceutical

use in manipulating the inflammatory response.

17. Document ID: US 5695993 A

L1: Entry 17 of 24

File: EPAB

Dec 9, 1997

PUB-NO: US005695993A

DOCUMENT-IDENTIFIER: US 5695993 A

TITLE: Cloning and regulation of an endothelial cell protein C/activated protein C receptor

PUBN-DATE: December 9, 1997

INVENTOR-INFORMATION:
NAME

FUKUDOME, KENJI

COUNTRY

US

ESMON, CHARLES T

US

INT-CL (IPC): C12N 5/16; C07H 21/04

EUR-CL (EPC): C07K014/705

AB: CHG DATE=19990617 STATUS=O>Human protein C and activated protein C were shown to bind to endothelium specifically, selectively and saturably (Kd=30 nM, 7000 sites per cell) in a Ca2+ dependent fashion. Expression cloning revealed a 1.3 kb CDNA that coded for a novel type

I transmembrane glycoprotein capable of binding protein C. This protein

18. Document ID: WO 9605303 A1

L1: Entry 18 of 24

File: EPAB

Feb 22, 1996

PUB-NO: WO009605303A1

DOCUMENT-IDENTIFIER: WO 9605303 A1

TITLE: CLONING AND REGULATION OF AN ENDOTHELIAL CELL PROTEIN C/ACTIVATED PROTEIN C RECEPTOR

PUBN-DATE: February 22, 1996

INVENTOR-INFORMATION:
NAME

FUKUDOME, KENJI

COUNTRY

N/A

ESMON, CHARLES T

N/A

INT-CL (IPC): C12N 15/12; C07K 14/705; A61K 39/395; C12N 15/11; A61K 38/17; C07K 16/28; G01N 33/68
EUR-CL (EPC): C07K014/705

AB: Human protein C and activated protein C were shown to bind to endothelium

specifically, selectively and saturably ($K_d=30$ nM, 7000 sites per cell) in a Ca^{2+} dependent fashion. Expression cloning revealed a 1.3 kb CDNA that coded for a novel type I transmembrane glycoprotein capable of binding protein C. This protein appears to be a member of the CD1/MHC superfamily. Like thrombomodulin, the receptor involved in protein C activation, the endothelial cell protein C receptor (EPCR) function and message are both down regulated by exposure of endothelium to TNF. Identification of EPCR as a member of the CD1/MHC superfamily provides insights into the role of protein C in regulating the inflammatory response, and determination of methods for pharmaceutical use in manipulating the inflammatory response.

L1: Entry 18 of 24

File: EPAB

Feb 22, 1996

DOCUMENT-IDENTIFIER: WO 9605303 A1
TITLE: CLONING AND REGULATION OF AN ENDOTHELIAL CELL PROTEIN C/ACTIVATED PROTEIN C RECEPTOR

FPAR:

Human protein C and activated protein C were shown to bind to endothelium specifically, selectively and saturably ($K_d=30$ nM, 7000 sites per cell) in a Ca^{2+} dependent fashion. Expression cloning revealed a 1.3 kb CDNA that coded for a novel type I transmembrane glycoprotein capable of binding protein C. This protein appears to be a member of the CD1/MHC superfamily. Like thrombomodulin, the receptor involved in protein C activation, the endothelial cell protein C receptor (EPCR) function and message are both down regulated by exposure of endothelium to TNF. Identification of EPCR as a member of the CD1/MHC superfamily provides insights into the role of protein C in regulating the inflammatory response, and determination of methods for pharmaceutical use in manipulating the inflammatory response.

19. Document ID: US 6037450 A

L1: Entry 19 of 24

File: DWPI

Mar 14, 2000

DERWENT-ACC-NO: 2000-246200
DERWENT-WEEK: 200023
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TITLE: Modified endothelial protein C receptor, useful as diagnostic marker of e.g. inflammation or autoimmune diseases, is a soluble or alternatively spliced form of the receptor

INVENTOR: ESMON, C T; KUROSAWA, S; STEARNS-KUROSAWA, D J

PRIORITY-DATA: 1997US-0884203 (June 27, 1997), 1998US-0082021 (May 20, 1998)

PATENT-FAMILY:
PUB-NO

PUB-DATE

LANGUAGE

PAGES

MAIN-IPC

US 6037450 A

March 14, 2000

N/A

024

C07K014/705

INT-CL (IPC): C07K 14/705

AB: NOVELTY - Modified endothelial protein C receptor (I) comprises amino acids (aa) 16-238 of a 238 aa sequence (S2) given in the specification, and has the C-terminal Cys replaced by some other aa, or is not palmitoylated., DETAILED DESCRIPTION - INDEPENDENT CLAIMS are also included for the following:, (1) isolated, natural alternatively spliced form (Ia) of (I), comprising (S2) with a protein insertion after Gly 201; and, (2) isolated, natural form (Ib) of (I) comprising residues 16-201 of (S2) that has been cleaved, in plasma, at a protease site before the transmembrane domain., ACTIVITY - None given., MECHANISM OF ACTION - (I) blocks activation of cellular protein C and anticoagulant activity of activated protein C., USE - (I) blocks activation of cellular protein C and anticoagulant activity of activated protein C. Measurement of (I), in standard immunoassays, is used to diagnose disorders involving coagulation or inflammation, or autoimmune diseases (e.g. lupus erythematosus; sepsis; diabetes; pre-eclampsia; restenosis and many others) also for monitoring treatment. (I) is a marker of endothelial cells so its presence may also indicate damage to large blood vessels, e.g. drug-induced damage. The antibodies used for immunoassay may also be used to characterize and isolate receptor proteins and to modulate receptor protein activity, particularly to inhibit binding to ligands.

L1: Entry 19 of 24

File: DWPI

Mar 14, 2000

DERWENT-ACC-NO: 2000-246200
DERWENT-WEEK: 200023
COPYRIGHT 2001 DERWENT INFORMATION LTD

TITLE: Modified endothelial protein C receptor, useful as diagnostic marker of e.g. inflammation or autoimmune diseases, is a soluble or alternatively spliced form of the receptor

ABTX:

NOVELTY - Modified endothelial protein C receptor (I) comprises amino acids (aa) 16-238 of a 238 aa sequence (S2) given in the specification, and has the C-terminal Cys replaced by some other aa, or is not palmitoylated.

20. Document ID: EP 1107790 A1, WO 200010609 A1, AU 9595013 A

L1: Entry 20 of 24

DERWENT-ACC-NO: 2000-224557
 DERWENT-WEEK: 200135
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TITLE: Specific delivery to nuclei of large vessel endothelial cells, useful for treatment or diagnosis of cardiovascular disease, by targeting the endothelial cell protein C receptor

INVENTOR: ESMON, C T; XU, J

PRIORITY-DATA: 1998US-0139425 (August 25, 1998)

PATENT-FAMILY:
 PUB-NO

	PUB-DATE	LANGUAGE	PAGES	MAIN-IPC
EP 1107790 A1	June 20, 2001	E	000	A61K045/06
WO 200010609 A1	March 2, 2000	E	023	A61K045/06
AU 9959013 A	March 14, 2000	N/A	000	A61K045/06

INT-CL (IPC): A61K 45/06

AB: NOVELTY - A method of selectively delivering molecules (I) to the nucleus of endothelial cells of large vessels by administering a conjugate (C) of (I) with an agent (II) that binds selectively to endothelial protein C receptor (EPCR)., DETAILED DESCRIPTION - An

INDEPENDENT CLAIM is also included for a conjugate of (I) which is not a diagnostic label, with either protein C, optionally activated, or an antibody reactive with EPCR, or fragments of it which bind EPCR., ACTIVITY - Antithrombotic; fibrinolytic; antiinflammatory; anticoagulant; vasoconstrictor., MECHANISM OF ACTION - EPCR translocates from the plasma membrane to the nucleus, and transports molecules that bind to it., USE - (C) are used to deliver therapeutic agents, e.g. nucleic acids, proteins, expression inhibitors, anti-inflammatories, anticoagulants, growth hormones etc., or diagnostic agents such as radiolabels, fluorescent labels and enzymatic labels, particularly in the cases of cardiovascular disease. Typical applications include preventing thrombosis, increasing fibrinolytic activity, or inhibiting leukocyte adhesion., ADVANTAGE - Since EPCR is present mainly on endothelial cells of large vessels, rather than similar cells in capillaries or other small vessels, especially in arteries, using it as target provides selective delivery of (I) with reduced risks of systemic complications. Serum stimulates nuclear translocation of EPCR, so delivery is enhanced during inflammatory or coagulative processes, often present in the treated subjects.

DERWENT-ACC-NO: 2000-224557
 DERWENT-WEEK: 200135
 COPYRIGHT 2001 DERWENT INFORMATION LTD

TITLE: Specific delivery to nuclei of large vessel endothelial cells, useful for treatment or diagnosis of cardiovascular disease, by targeting the endothelial cell protein C receptor

ABTX:

NOVELTY - A method of selectively delivering molecules (I) to the nucleus of endothelial cells of large vessels by administering a conjugate (C) of (I) with an agent (II) that binds selectively to endothelial protein C receptor (EPCR).

ABTX:

DETAILED DESCRIPTION - An INDEPENDENT CLAIM is also included for a conjugate of (I) which is not a diagnostic label, with either protein C, optionally activated, or an antibody reactive with EPCR, or fragments of it which bind EPCR.

ABTX:

MECHANISM OF ACTION - EPCR translocates from the plasma membrane to the nucleus, and transports molecules that bind to it.

ABTX:

ADVANTAGE - Since EPCR is present mainly on endothelial cells of large vessels, rather than similar cells in capillaries or other small vessels, especially in arteries, using it as target provides selective delivery of (I) with reduced risks of systemic complications. Serum stimulates nuclear translocation of EPCR, so delivery is enhanced during inflammatory or coagulative processes, often present in the treated subjects.

21. Document ID: EP 1033031 A1, DE 19751465 A1, WO 9927706 A1, DE 19751465 C2

DERWENT-ACC-NO: 1999-328210
 DERWENT-WEEK: 200044
 COPYRIGHT 2001 DERWENT INFORMATION LTD

TITLE: Sensitivity values determination unit for copying images taken by digital camera

INVENTOR: FINDEIS, G; FUERSICH, M ; KEUPP, W

PRIORITY-DATA: 1997DE-1051465 (November 20, 1997)

PATENT-FAMILY:
 PUB-NO

	PUB-DATE	LANGUAGE	PAGES	MAIN-IPC
EP 1033031 A1	September 6, 2000			

G
000
H04N001/60
DE 19751465 A1
May 27, 1999
N/A
005
H04N001/40
WO 9927706 A1
June 3, 1999
G
000
H04N001/60
DE 19751465 C2
September 2, 1999
N/A
000
H04N001/40

INT-CL (IPC): H04N 1/32; H04N 1/40; H04N 1/60

AB: NOVELTY - The unit comprises a recognition module (EP,CR) of the type of digital camera (KT1...N) which has taken the image. A control (CR) determines the sensitivity values in dependence on the recognized type. There is a memory (SP2) for several copying data sets

(GD1...N), containing sensitivity values for image copying from different camera types.,

DETAILED DESCRIPTION - An INDEPENDENT CLAIM is included for a determination method., USE - For

fotoprinter, minilab or computer controlled printer., ADVANTAGE - Precise and naturally true

reproduction of images taken by digital camera., DESCRIPTION OF DRAWING(S) - The figure

presents example of the unit., recognition module EP,CR, camera type KT, control CR, copying data sets. GD

L1: Entry 21 of 24

File: DWPI

Sep 6, 2000

DERWENT-ACC-NO: 1999-328210
DERWENT-WEEK: 200044
COPYRIGHT 2001 DERWENT INFORMATION LTD

TITLE: Sensitivity values determination unit for copying images taken by digital camera

ABTX:

NOVELTY - The unit comprises a recognition module (EP,CR) of the type of digital camera (KT1...N) which has taken the image. A control (CR) determines the sensitivity values in dependence on the recognized type. There is a memory (SP2) for several copying data sets (GD1...N), containing sensitivity values for image copying from different camera types.

ABTX:

recognition module EP,CR

22. Document ID: JP 2001508876 W, US 5804392 A, WO 9900673 A1, AU 9882694 A, EP 991946 A1

L1: Entry 22 of 24

File: DWPI

Jul 3, 2001

DERWENT-ACC-NO: 1998-505645
DERWENT-WEEK: 200142
COPYRIGHT 2001 DERWENT INFORMATION LTD

TITLE: Immuno-based detection of protein C receptor - useful in the diagnosis of inflammatory and coagulation states and disorders associated with damage to endothelium and large blood vessel disease

INVENTOR: ESMON, C T; KUROSAWA, S ; STEARNS-KUROSAWA, D J

PRIORITY-DATA: 1997US-0884203 (June 27, 1997)

PATENT-FAMILY:
PUB-NO

PUB-DATE

LANGUAGE
PAGES
MAIN-IPC

JP 2001508876 W
July 3, 2001

N/A

058

G01N033/53

US 5804392 A
September 8, 1998

N/A

023

G01N033/53

WO 9900673 A1
January 7, 1999

E

000

G01N033/68

AU 9882694 A
January 19, 1999

N/A

000

G01N033/68

EP 991946 A1
April 12, 2000

E

000

G01N033/68

INT-CL (IPC): C07K 14/705; C07K 16/28; G01N 33/53; G01N 33/564; G01N 33/68

AB: An assay for soluble endothelial protein C receptor comprises containing a sample from a patient to be tested and measuring the amount of soluble endothelial protein C receptor., Also claimed is a kit for detection and measurement of endothelial protein C receptor comprising:, (a) an antibody immunoreactive with endothelial protein C receptor in a patient sample; and, (c) standards to correlate the amount of reaction to normal and abnormal levels of endothelial protein C receptor., USE - The assay is used for the diagnosis of coagulation and inflammatory states and disorders, damage to endothelium, and large blood vessel disease, e.g. autoimmune diseases, transplantation, sepsis, shock, pre-eclampsia, diabetes, vascular disease (especially cardiopulmonary bypass, unstable angina, restenosis and angioplasty), kidney disease and liver disease (claimed). Protein C is involved in the regulation of a host response to inflammation. The protein is one of the last components to be activated in the coagulation system, and is thought to control coagulation and inflammation. Activation of the receptor through a pathway involving thrombin, activates protein C. The protein C

pathway is apparently only involved in large blood vessels, not capillaries, and so is activated with for major vascular conditions, and the increased presence of the receptor in the conditions stated makes it ideal as a diagnostic component.

L1: Entry 22 of 24

File: DWPI

Jul 3, 2001

DERWENT-ACC-NO: 1998-505645
DERWENT-WEEK: 200142
COPYRIGHT 2001 DERWENT INFORMATION LTD

TITLE: Immuno-based detection of protein C receptor - useful in the diagnosis of inflammatory and coagulation states and disorders associated with damage to endothelium and large blood vessel disease

ABTX:

An assay for soluble endothelial protein C receptor comprises containing a sample from a patient to be tested and measuring the amount of soluble endothelial protein C receptor.

ABTX:

Also claimed is a kit for detection and measurement of endothelial protein C receptor comprising:

ABTX:

(a) an antibody immunoreactive with endothelial protein C receptor;

ABTX:

(b) reagents to detect a reaction between the Ab and endothelial protein C receptor in a patient sample; and

ABTX:

(c) standards to correlate the amount of reaction to normal and abnormal levels of endothelial protein C receptor.

23. Document ID: US 6200751 B1, WO 9820041 A1, AU 9854317 A, EP 937104 A1, AU 719629 B, JP 2001503273 W

L1: Entry 23 of 24

File: DWPI

Mar 13, 2001

DERWENT-ACC-NO: 1998-286871
DERWENT-WEEK: 200120
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TITLE: Regulatory elements from the endothelial protein C receptor promoter - useful to direct expression of genes or nucleotide molecules e.g. to endothelial cells or only large vessel endothelial cells in gene therapy

INVENTOR: ESMON, C T; GU, J

PRIORITY-DATA: 1997US-0054533 (August 4, 1997), 1996US-0030718 (November 8, 1996), 1997US-0965729 (November 7, 1997)

PATENT-FAMILY:

PUB-NO	PUB-DATE	LANGUAGE	PAGES	MAIN-IPC
US 6200751 B1	March 13, 2001	N/A	000	C12Q001/68
WO 9820041 A1	May 14, 1998	E	069	C07K014/705
AU 9854317 A	May 29, 1998	N/A	000	C07K014/705
EP 937104 A1	August 25, 1999	E	000	C07K014/705
AU 719629 B	May 11, 2000	N/A	000	C07K014/705
JP 2001503273 W	March 13, 2001	N/A	028	C12N015/09

INT-CL (IPC): C07H 21/04; C07K 14/705; C12N 15/09; C12N 15/12; C12P 21/02; C12Q 1/68

AB: Regulatory elements (I) isolated from the endothelial protein C receptor (EPCR) promoter which directs expression selectively to endothelial cells are new. Also claimed are constructs for heterologous gene expression comprising (I), USE - The regulatory elements are useful to control expression of a gene/biologically active nucleotide molecule (claimed), by expressing these under control of one of the elements (optionally with the thrombin response element) (claimed). Expression of the gene/nucleotide molecule is selectively in large vessel endothelial cells and/or as a result of environmental stimuli (either thrombin or serum) can be achieved by inclusion of the appropriate regulatory element(s). Atherosclerosis and most other vascular diseases primarily occur in large vessels, and for gene therapy for such diseases it is desirable to target endothelial cells, the primary defence mechanism against cellular infiltration and thrombosis. The constructs are therefore particularly useful in gene therapy, especially when the gene encodes a protein, or the nucleotide molecules are antisense, triplex forming, ribozymes or guide sequences for RNAase P (claimed) which are used to mutate or stop transcription of a particular gene. Such genes/nucleotide molecules may be expressed in vivo in patients or in cell culture (claimed). For example, endothelial response elements may be used for any gene therapy where systemic distribution is required, whilst large vessel endothelial cell response elements are useful for expression of thrombomodulin in large vessel endothelium to decrease clot propensity at atheromas or in autoimmune diseases; the environmental stimuli response element(s) are useful e.g. to deliver agents whose expression

should be increased during increased thrombin/platelet activation or regional trauma. The regulatory elements are also useful as hybridisation probes, in increasing expression of recombinant proteins by exposure of the encoding construct to thrombin and in drug screening and design (not claimed).

Regulatory elements (I) isolated from the endothelial protein C receptor (EPCR) promoter which directs expression selectively to endothelial cells are new. Also claimed are constructs for heterologous gene expression comprising (I), USE - The regulatory elements are useful to control expression of a gene/biologically active nucleotide molecule (claimed), by expressing these under control of one of the elements (optionally with the thrombin response element) (claimed). Expression of the gene/nucleotide molecule is selectively in large vessel endothelial cells and/or as a result of environmental stimuli (either thrombin or serum) can be achieved by inclusion of the appropriate regulatory element(s). Atherosclerosis and most other vascular diseases primarily occur in large vessels, and for gene therapy for such diseases it is desirable to target endothelial cells, the primary defence mechanism against cellular infiltration and thrombosis. The constructs are therefore particularly useful in gene therapy, especially when the gene encodes a protein, or the nucleotide molecules are antisense, triplex forming, ribozymes or guide sequences for RNAase P (claimed) which are used to mutate or stop transcription of a particular gene. Such genes/nucleotide molecules may be expressed in vivo in patients or in cell culture (claimed). For example, endothelial response elements may be used for any gene therapy where systemic distribution is required, whilst large vessel endothelial cell response elements are useful for expression of thrombomodulin in large vessel endothelium to decrease clot propensity at atheromas or in autoimmune diseases; the environmental stimuli response element(s) are useful e.g. to deliver agents whose expression should be increased during increased thrombin/platelet activation or regional trauma. The regulatory elements are also useful as hybridisation probes, in increasing expression of recombinant proteins by exposure of the encoding construct to thrombin and in drug screening and design (not claimed).

L1: Entry 23 of 24

File: DWPI

Mar 13, 2001

DERWENT-ACC-NO: 1998-286871
DERWENT-WEEK: 200120
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TITLE: Regulatory elements from the endothelial protein C receptor promoter - useful to direct expression of genes or nucleotide molecules e.g. to endothelial cells or only large vessel endothelial cells in gene therapy

ABTX:
Regulatory elements (I) isolated from the endothelial protein C receptor (EPCR) promoter which directs expression selectively to endothelial cells are new. Also claimed are constructs for heterologous gene expression comprising (I)

ABEQ:
Regulatory elements (I) isolated from the endothelial protein C receptor (EPCR) promoter which

directs expression selectively to endothelial cells are new. Also claimed are constructs for heterologous gene expression comprising (I)

24. Document ID: AU 707349 B, WO 9605303 A1, AU 9532723 A, EP 777731 A1, US 5695993 A, US 5852171 A

L1: Entry 24 of 24

File: DWPI

Jul 8, 1999

DERWENT-ACC-NO: 1996-139699
DERWENT-WEEK: 199938
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TITLE: Isolated endothelial cell protein C/activated protein C receptor - used to inhibit inflammatory responses, screen for cpds. which alter receptor binding and, by blocking receptor binding, enhance inflammatory response

INVENTOR: ESMON, C T; FUKUDOME, K

PRIORITY-DATA: 1994US-0289699 (August 12, 1994),
1997US-0878283 (June 18, 1997)

PATENT-FAMILY:
PUB-NO

PUB-DATE	LANGUAGE	PAGES	MAIN-IPC
AU 707349 B	July 8, 1999	N/A	000
WO 9605303 A1	February 22, 1996	E	058
AU 9532723 A	March 7, 1996	N/A	000
EP 777731 A1	June 11, 1997	E	000
US 5695993 A	December 9, 1997	N/A	028
US 5852171 A	December 22, 1998	N/A	000

INT-CL (IPC): A61K 38/17; A61K 39/395; C07H 21/04; C07K 14/705; C07K 16/28; C12N 5/16; C12N 15/11; C12N 15/12; G01N 33/68

AB: Isolated endothelial cell protein C/activated protein C receptor (EPCR) is new. Also

claimed are: (1) a nucleotide sequence encoding EPCR; and (2) an antibody or fragment specifically immunoreactive with a unique epitope of EPCR., USE - EPCR and substances which up-regulate its expression are useful to inhibit inflammatory responses (claimed). This inhibition is useful in the treatment of, e.g. Gram-negative sepsis, stroke, thrombosis, septic shock, ARDS and pulmonary emboli. EPCR is also useful to screen for cpds. which alter its binding to (activated) protein C (claimed). Localising EPCR to surfaces in contact with blood will render the surfaces anticoagulant as EPCR binds and concentrates the anticoagulant activated protein C at the surface. Its function can also be enhanced by overexpressing EPCR in endothelium that could be used to coat vascular grafts in patients with vascular disease, or in stents in cardiac patients. Using blocking cpds. to prevent (activated) protein C binding to EPCR it is possible to enhance an inflammatory response and so treat solid tumours., Isolated endothelial cell protein C/activated protein C receptor (EPCR) is new. Also claimed are: (1) a nucleotide sequence encoding EPCR; and (2) an antibody or fragment specifically immunoreactive with a unique epitope of EPCR., USE - EPCR and substances which up-regulate its expression are useful to inhibit inflammatory responses (claimed). This inhibition is useful in the treatment of, e.g. Gram-negative sepsis, stroke, thrombosis, septic shock, ARDS and pulmonary emboli. EPCR is also useful to screen for cpds. which alter its binding to (activated) protein C (claimed). Localising EPCR to surfaces in contact with blood will render the surfaces anticoagulant as EPCR binds and concentrates the anticoagulant activated protein C at the surface. Its function can also be enhanced by overexpressing EPCR in endothelium that could be used to coat vascular grafts in patients with vascular disease, or in stents in cardiac patients. Using blocking cpds. to prevent (activated) protein C binding to EPCR it is possible to enhance an inflammatory response and so treat solid tumours., Isolated endothelial cell protein C/activated protein C receptor (EPCR) is new. Also claimed are: (1) a nucleotide sequence encoding EPCR; and (2) an antibody or fragment specifically immunoreactive with a unique epitope of EPCR., USE - EPCR and substances which up-regulate its expression are useful to inhibit inflammatory responses (claimed). This inhibition is useful in the treatment of, e.g. Gram-negative sepsis, stroke, thrombosis, septic shock, ARDS and pulmonary emboli. EPCR is also useful to screen for cpds. which alter its binding to (activated) protein C (claimed). Localising EPCR to surfaces in contact with blood will render the surfaces anticoagulant as EPCR binds and concentrates the anticoagulant activated protein C at the surface. Its function can also be enhanced by overexpressing EPCR in endothelium that could be used to coat vascular grafts in patients with vascular disease, or in stents in cardiac patients. Using blocking cpds. to prevent (activated) protein C binding to EPCR it is possible to enhance an inflammatory response and so treat solid tumours.

L1: Entry 24 of 24

File: DWPI

Jul 8, 1999

DERWENT-ACC-NO: 1996-139699
DERWENT-WEEK: 199938

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TITLE: Isolated endothelial cell protein C/activated protein C receptor - used to inhibit inflammatory responses, screen for cpds. which alter receptor binding and, by blocking receptor binding, enhance inflammatory response

ABTX:

Isolated endothelial cell protein C/activated protein C receptor (EPCR) is new. Also claimed are: (1) a nucleotide sequence encoding EPCR; and (2) an antibody or fragment specifically immunoreactive with a unique epitope of EPCR.

ABTX:

USE - EPCR and substances which up-regulate its expression are useful to inhibit inflammatory responses (claimed). This inhibition is useful in the treatment of, e.g. Gram-negative sepsis, stroke, thrombosis, septic shock, ARDS and pulmonary emboli. EPCR is also useful to screen for cpds. which alter its binding to (activated) protein C (claimed). Localising EPCR to surfaces in contact with blood will render the surfaces anticoagulant as EPCR binds and concentrates the anticoagulant activated protein C at the surface. Its function can also be enhanced by overexpressing EPCR in endothelium that could be used to coat vascular grafts in patients with vascular disease, or in stents in cardiac patients. Using blocking cpds. to prevent (activated) protein C binding to EPCR it is possible to enhance an inflammatory response and so treat solid tumours.

ABEQ:

Isolated endothelial cell protein C/activated protein C receptor (EPCR) is new. Also claimed are: (1) a nucleotide sequence encoding EPCR; and (2) an antibody or fragment specifically immunoreactive with a unique epitope of EPCR.

ABEQ:

USE - EPCR and substances which up-regulate its expression are useful to inhibit inflammatory responses (claimed). This inhibition is useful in the treatment of, e.g. Gram-negative sepsis, stroke, thrombosis, septic shock, ARDS and pulmonary emboli. EPCR is also useful to screen for cpds. which alter its binding to (activated) protein C (claimed). Localising EPCR to surfaces in contact with blood will render the surfaces anticoagulant as EPCR binds and concentrates the anticoagulant activated protein C at the surface. Its function can also be enhanced by overexpressing EPCR in endothelium that could be used to coat vascular grafts in patients with vascular disease, or in stents in cardiac patients. Using blocking cpds. to prevent (activated) protein C binding to EPCR it is possible to enhance an inflammatory response and so treat solid tumours.

ABEQ:

Isolated endothelial cell protein C/activated protein C receptor (EPCR) is new. Also claimed are: (1) a nucleotide sequence encoding EPCR; and (2) an antibody or fragment specifically immunoreactive with a unique epitope of EPCR.

ABEQ:

USE - EPCR and substances which up-regulate its expression are useful to inhibit inflammatory responses (claimed). This inhibition is useful in the treatment of, e.g. Gram-negative sepsis, stroke, thrombosis, septic shock, ARDS and pulmonary emboli. EPCR is also useful to screen for cpds. which alter its binding to (activated) protein C (claimed). Localising EPCR to surfaces in contact with blood will render the surfaces anticoagulant as EPCR binds and concentrates the anticoagulant

activated protein C at the surface. Its function can also be enhanced by overexpressing EPCR in endothelium that could be used to coat vascular grafts in patients with vascular disease, or in stents in cardiac patients. Using blocking cpds. to prevent (activated) protein C binding to EPCR it is possible to enhance an inflammatory response and so treat solid tumours.